

# FINAL REPORT

Sustainable Range Management of RDX and TNT by  
Phytoremediation with Engineered Plants

SERDP Project ER-1498

APRIL 2016

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## List of Acronyms

ADNT	amino-dinitrotoluene
ALA	$\alpha$ -aminolevuleic acid
BAP	6-benzylaminopurine
cDNA	complimentary DNA
CaMV	cauliflower mosaic virus
2,4-D	2,4-dichlorophenoxyacetic acid
DMF	dimethylformamide
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetraacetic acid
FAD	flavin adenine dinucleotide
GA	gibberellic acid
GFP	green fluorescent protein
GSH	glutathione
GST	glutathione-S-transferase
GTase	UDP-glycosyltransferase
GUS	$\beta$ -glucuronidase
HADNT	hydroxylaminodinitrotoluene
HMX	high melting explosive (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine)
HPLC	high performance liquid chromatography
LB	lysogeny broth
MEDINA	methylenedinitramine
MDA	3,4-methylenedioxyamphetamine
MDHAR	monodehydroascorbate reductase
MS	mass spectrometry
NAA	1-naphthaleneacetic acid
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
<i>nfsI</i>	gene encoding TNT-detoxifying nitroreductase from <i>Enterobacter cloacae</i>
PCR	polymerase chain reaction
PETN	pentaerythritol tetranitrate
PMSF	phenylmethanesulfonylfluoride
ppb	parts per billion
PVPP	polyvinylpolypyrrolidone
RDX	hexahydro-1,3,5-trinitro-1,3,5-triazine (Royal demolition explosive)
qRT-PCR	quantitative real-time PCR
rpm	revolutions per minute
ROMT	culture medium
SE	standard error of the mean
SD	standard deviation
TNT	2,4,6-trinitrotoluene
UTR	untranslated region
XplA	RDX-degrading enzyme from <i>Rhodococcus rhodochrous</i> 11Y

XplB

Reductase encoded adjacently to *xplA* in *R. rhodochrous*

## **Keywords**

Phytoremediation, RDX, TNT, Arabidopsis, wheatgrass, switchgrass, XplA, Explosives

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## Abstract

### Objectives

Decades of military activity on live-fire training ranges have resulted in the contamination of land and groundwater by recalcitrant high explosives, particularly TNT and RDX. TNT and its transformation products are highly toxic, but tend to bind strongly to clay and organic matter in soil, so are largely contained at the site of contamination. RDX, however, poses a major concern, because of its high mobility through the soil water table and consequent potential for contamination of groundwater. RDX contamination of training ranges is now proving to be a significant threat to drinking water sources. Currently, there are no cost-effective processes to contain RDX or remediate large areas of contaminated land on training ranges. The project responds directly to specific research objectives stated in SON number CUSON-06-04 including: i) sustained prevention of migration of surface and near surface soil contamination by energetic materials; ii) development of technologies applicable to large, potentially vegetated areas, directed at the long-term control of energetic materials through repeated applications; iii) sustainable technology; iv) inexpensive rapidly deployable technology; and v) adaptable to an unpredictable influx of contaminants.

The objective of this project was to engineer transgenic grasses to remove and degrade RDX in the root zone of soil contaminated with explosives. The expression in plants of a novel, RDX-degrading cytochrome P450 gene, *xplA*, was investigated. This enzyme, along with its redox partner (XplB), can degrade RDX to harmless metabolites. Since TNT often occurs as a co-contaminant alongside RDX, it was also necessary to engineer resistance to TNT, because this explosive is highly toxic to plants.

### Technical Approach

Under a previous SERDP project (ER-1318), we demonstrated that expression of the rhodococcal gene, *xplA*, in the model plant *Arabidopsis*, conferred the ability to tolerate and degrade high concentrations of RDX. We also showed that expression of the bacterial nitroreductase gene, *nfsI*, in tobacco and *Arabidopsis* conferred resistance to toxic levels of TNT.

The research described for project ER-1498 used a variety of different approaches to improve and extend the capacity of plants to detoxify RDX and TNT, while addressing some of the practical and regulatory issues involved in the development of this technology. For example, attempts were made to improve the RDX-degrading efficiency of the XplA protein itself. In other work, we tried to improve the efficiency of the nitroreductase enzyme by targeting its expression specifically to root tissues. Microarray studies and mutant screens were used to identify additional genes involved in TNT detoxification.

Efforts were made to address the problem of biocontainment of transgenes, using a chloroplast transformation approach in tobacco. A bioassay system was used to investigate herbivory of transgenic and RDX-treated *Arabidopsis* plants.

Model plant systems, such as *Arabidopsis* and tobacco, were used to develop the technologies, because of their efficient transformation methods and well-developed genomics tools. However, the major objective of this project was to generate transgenic plants that can phytoremediate TNT and RDX pollution in field conditions, on training ranges. For this purpose, plants need to be robust enough to withstand fire and tolerate the effects of disruption by heavy machinery. Hence, we aimed to introduce the *xplA/B* and *nfsI* genes into suitable grass species that could be grown on range land, in order to decontaminate soil over a large area. The effectiveness of RDX-removal by the transgenic grasses would be tested in demonstration-scale field experiments.

### Results

Rational engineering based on the structure of XplA and random mutagenesis and directed evolution techniques were used to characterize XplA, with the aim of improving the ability of

the enzyme to degrade RDX. A possible mode of action for denitration of RDX was suggested.

Liquid culture and soil experiments confirmed that Arabidopsis plants containing the transgenes *xplA/B* and *nfsI* could remove and degrade RDX from soil and leachate in the presence of TNT.

In microarray experiments, members of several candidate gene families were up-regulated in response to TNT. These included GTases (UDP-glycosyltransferases), OPRs (oxophytodienoate reductases), GSTs (glutathione-S-transferases) and cytochromes P450. Arabidopsis plants engineered to over-express GTases, OPRs or GSTs were more tolerant to TNT than wild-type. Production of different TNT-glutathione conjugates was investigated, in the context of possible manipulation of GSTs to optimize TNT-detoxification capacity.

Mutant screens identified an additional gene (*MDHAR6*), whose loss-of-function conferred resistance to TNT, as confirmed by liquid culture and soil-based experiments. Accumulation in the mitochondria of superoxide produced by MDHAR6 with TNT as substrate was proposed as the main mechanism for TNT toxicity in plants.

Experiments were carried out with tissue-specific promoters, whereby *nfsI* was targeted to the roots of Arabidopsis, using a root-specific promoter (*RolD*). Liquid culture experiments and soil studies showed that the transformed plants had increased tolerance to TNT, suggesting that such promoters could be a useful candidate for targeted transgene expression in dicot species.

Attempts were made to address some of the regulatory and public acceptance issues surrounding the release of transgenic plants. For example, experiments were carried out to engineer biocontainment of transgenes by chloroplast transformation. The protocol and vectors were optimized for successful introduction of *xplA/B* and *nfsI* into tobacco chloroplasts. This conferred tolerance to TNT and improved TNT uptake from solution, although the capacity of the plants to take up and degrade RDX was no better than wild-type. Studies were undertaken to investigate whether a generalist insect herbivore (the locust *Schistocerca gregaria*) showed any feeding preference for or against Arabidopsis plants transformed with *xplA/B* and/or treated with RDX. The locusts showed no preference for any of the treatment combinations, compared with wild-type. In other experiments, attempts to assess the impact of the genetically modified plants on soil microflora showed that expression of *nfsI* by tobacco plants did not have a non-target effect on soil microbial communities.

Western wheatgrass and switchgrass were chosen as the most suitable grasses to engineer for phytoremediation purposes on range land. Creeping bentgrass was used as a model to develop the technology. Tissue culture methods, transformation protocols and regeneration techniques were developed and optimized during the course of the project. The *xplA/B* and *nfsI* genes were successfully transferred into the above grasses, using a range of newly-designed vectors with monocot promoters. Transformed switchgrass was able to remove and degrade RDX from liquid culture medium. Preliminary experiments suggested that transgenic switchgrass could also remove RDX from soil leachate.

Methods for seeding grasses on ranges were developed, using seedballs to seed remote areas. Seedball construction and formulation were optimized to improve germination and establishment.

Field studies were started, to validate the technology at demonstration scale. The optimal spacing of the grass plants in the trials was determined. Test cells were designed and constructed at Fort Drum (NY, USA) for the outdoor trials and these trials are about to commence in May 2016.

## Benefits

The aim of this project was to develop transgenic grasses with unique abilities to detoxify TNT and degrade RDX. Such plants have the potential to provide a self-sustaining,

inexpensive and environmentally-friendly method of range restoration that can be used over large areas of land for preventing groundwater contamination. Engineering plants to remove explosives could provide an efficacious means to clean up land contaminated through military activities, and presents an effective potential solution to a serious environmental problem.

## Objective

Decades of military activity on live-fire training ranges have resulted in the contamination of land and groundwater by recalcitrant high explosives, particularly hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and 2,4,6-trinitrotoluene (TNT). TNT and its transformation products are highly toxic, but tend to bind strongly to clay and organic matter in soil, so are largely contained at the site of contamination. RDX, however, poses a major concern, because of its high mobility through the soil water table and consequent potential for contamination of groundwater. RDX contamination of training ranges is now proving to be a significant threat to drinking water sources, such as those close to the Massachusetts Military Reservation (Claussen *et al.*, 2006). These factors mean that RDX presents an immediate and future health problem to society. Currently, there are no cost-effective processes to contain RDX or remediate large areas of contaminated land on training ranges.

The objective of this project was to engineer transgenic grasses to remove and degrade RDX in the root zone of soil contaminated with explosives. The expression in plants of a novel, RDX-degrading cytochrome P450 gene, *xplA*, was investigated. This enzyme, along with its redox partner (XplB), can degrade RDX to harmless metabolites. Since TNT often occurs as a co-contaminant alongside RDX, it was also necessary to engineer resistance to TNT, because this explosive is highly toxic to plants.

## Background

RDX is readily taken up by plant roots and translocated to the aerial tissues of plants (Vila *et al.*, 2007). It is reduced to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine and hexahydro-1,3-nitroso-5-nitro-1,3,5-triazine in the leaves, with subsequent mineralization of the heterocyclic ring requiring light (Van Aken *et al.*, 2004). However, despite high uptake rates, plants have inherently low ability to degrade RDX (Best *et al.*, 1999; Winfield *et al.*, 2004). Microorganisms with the ability to degrade RDX have been isolated, including *Rhodococcus rhodochrous* strain 11Y (Seth-Smith *et al.*, 2002). The RDX-degrading ability of this bacterium, encoded by *xplA*, has been shown to be due to a cytochrome P450, which catalyses the degradation of RDX. Arabidopsis plants expressing XplA have been shown to remove RDX from solution (Rylott *et al.*, 2006), suggesting that this approach could be successfully incorporated into a phytoremediation programme. Soil studies have shown that plant biomass is increased in XplA-expressing plants grown on RDX-contaminated soil, compared with uncontaminated soil, indicating that XplA-expressing plants can utilize the nitrite released from the degradation of RDX as a nitrogen source for growth (Rylott *et al.*, 2006). Plants co-expressing XplA and XplB, the partnering reductase for XplA in *Rhodococcus*, showed an additional increase in the rate of RDX removal (Jackson *et al.*, 2007).

TNT is toxic to all organisms tested so far, including plants (Rocheleau *et al.*, 2006). Unlike RDX, TNT binds tightly to the humic fraction of soil (Hundal *et al.*, 1997, Thorn & Kennedy, 2002). TNT is most commonly biotransformed in soil by type I bacterial nitroreductases (NRs) to hydroxylamino dinitrotoluenes (HADNTs), with subsequent reduction to amino dinitrotoluenes (ADNTs). Plants have only a limited ability to detoxify TNT (Gandia-Herrero *et al.*, 2008, Rylott and Bruce, 2009). To overcome this, bacterial genes conferring TNT detoxification activity have been engineered into plants. The *onr* gene encoding pentaerythritol tetranitrate reductase and the *nfs1* gene encoding a nitroreductase (NR) from

*Enterobacter cloacae* have both been independently expressed in tobacco (French *et al.*, 1999; Hannink *et al.*, 2001). The resulting plant lines had increased capacities for tolerating and detoxifying TNT.

The high mobility of RDX means that it needs to be intercepted on military ranges before it leaches into the ground water. Plants, with their extensive root systems, offer a potentially viable and sustainable means of attenuating this pollutant. RDX and TNT are often found together on contaminated sites; hence if RDX is to be effectively removed from the environment, plants need to be engineered to tolerate the toxicity of the co-polluting TNT.

The overall aim of the research described in this report was to transfer the above technology from model plant species into grass species that would be suitable for phytoremediating contaminated military land on a large scale. Transgenic grasses would be engineered to express the *xplA/B* and *nfsI* genes discussed above, and their efficacy of removal of RDX from contaminated soil tested in glasshouse and then through field experiments supported by ESTCP.

The following sections provide a task-by-task description of the materials and methods used and the results obtained. The project tasks are summarized in Table 1, below.

Task	Project tasks and sub tasks	Start year	Planned Completion	Revised Completion	Actual Completion
1	Characterization of XplA and variants	2006	3/2007	6/2008	3/2009
1.1	Purification and characterization of XplA	2006	3/2007		3/2007
1.2	Generation of <i>xplA</i> mutants	2006	3/2007		12/2008
2	Identification of TNT active GTases	2006	10/2006		10/2006
3	Selection of range grasses	2006	3/2007		3/2007
4	Development of methods for transformation of grasses	2006	3/2008		3/2008
5	Multiple transgenics: expression of NR, XplA or XplA variants and GTases in Arabidopsis	2007	3/2010	7/2012	5/2015
6	Transformation of grasses	2006	3/2010	7/2012	11/2014
6.1	Characterization of transformed grasses	2007	3/2010	3/2013	
7	Analysis of tissue-specific promoters	2007	3/2009	7/2012	
8	Soil studies with transgenic grasses	2008	3/2010		7/2015
9	Engineer XplA HMX-degrading Arabidopsis plant lines	2008	3/2009	3/2009	
10	Selection of explosive-transforming grasses	2009	3/2010	3/2013	3/2013
10.1	Increase rates of RDX degradation by transgenic western wheatgrass lines	2010	3/2012		7/2015
10.2	Engineer transgenic wheatgrass to degrade RDX and detoxify TNT	2010	3/2013		7/2015
11	Perform greenhouse studies to validate the technology	2010	7/2013		7/2015
12	Engineer biocontainment of transgenes	2010	7/2013		7/2013
13	Develop methods for seedling grasses on ranges	2010	3/2013		7/2010
14	Monitor insect herbivoryon genetically modified plants	2011	7/2012		1/2012
15	Assess the impact of genetically modified plants on soil microflora	2012	3/2013		3/2013

**Table 1 Project tasks and sub-tasks for ER-1498**

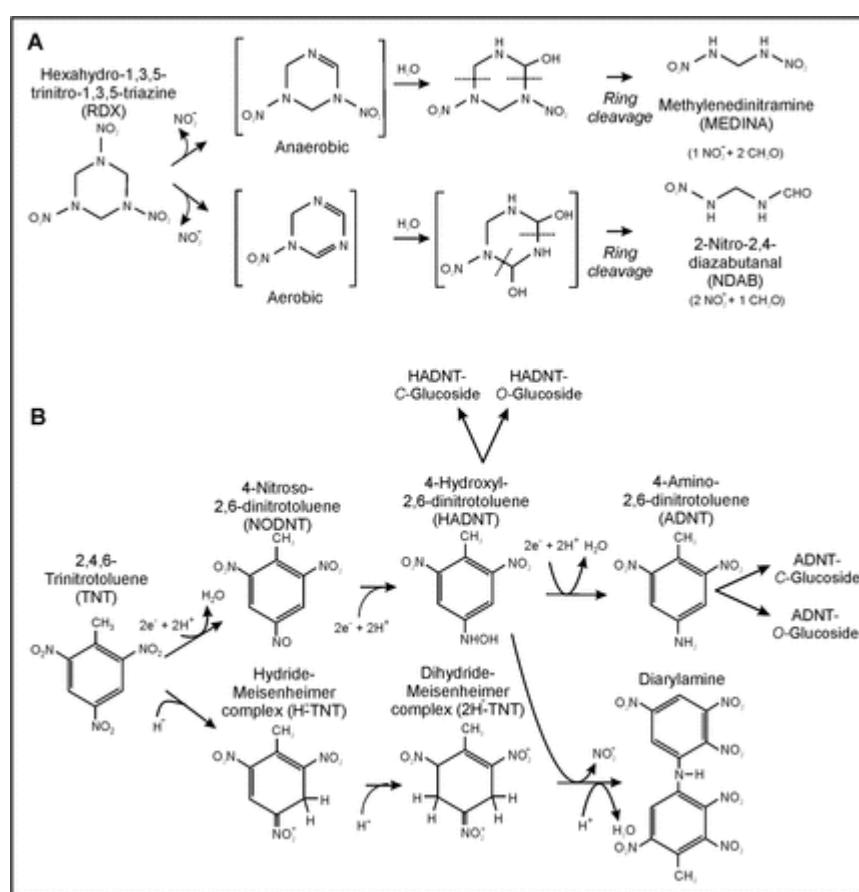
## Materials & Methods, Results & Discussion

### Task 1

#### Characterization of XplA and variants

In a previous SERDP-funded project (ER-1318), it was demonstrated that expression in the model plant *Arabidopsis* of a bacterial cytochrome P450 enzyme, known as XplA, conferred the ability to tolerate and degrade high concentrations of RDX.

As described previously (Seth-Smith *et al.*, 2002), a bacterium, *Rhodococcus rhodochrous* (strain 11Y) was initially isolated from an RDX-contaminated site and was able to grow on RDX as its sole nitrogen source. A gene cluster in strain 11Y essential for RDX degradation was identified and shown to contain a novel cytochrome P450, termed XplA, and a redox partner, XplB, which together were capable of catalysing the biotransformation of RDX *in vitro*. Products of biotransformation were shown to be nitrite and formaldehyde and (under anaerobic conditions) methylenedinitramine (MEDINA) or (under aerobic conditions) 4-nitro-2,4-diazabutanal (NDAB) (Figure 1.1).



**Figure 1.1**

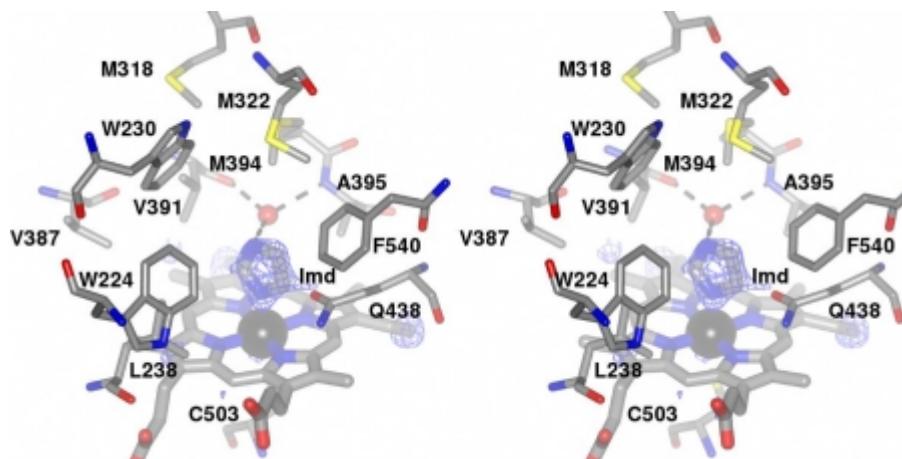
**The biochemical pathways for detoxification of RDX and TNT.** (A) Bacterial degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in bacteria. Dotted lines indicate position of ring cleavage, compounds in brackets are hypothetical (adapted from Jackson *et al.*, 2007). (B) Transformation of 2,4,6-trinitrotoluene (TNT). Diarylamine identified in bacteria only, conjugates in plants only (Rylott *et al.*, 2011a, Rylott *et al.*, 2015).

Our studies revealed that the structural organization of XplA is unique, comprising a heme-domain, C-terminally fused to its native flavodoxin redox partner (Rylott *et al.*, 2006).

In order to find out more about the molecular mechanism of substrate specificity in XplA, we performed experiments to elucidate the structure of the heme domain of the enzyme. Detailed

materials and methods are presented in Sabbadin *et al.*, (2009). The XplA-heme domain was cloned, expressed in *E.coli*, purified and crystallized. Full length XplA was also expressed and purified, as described in Jackson *et al.*, (2007) and crystallized as described in Sabbadin *et al.*, (2009). X-ray diffraction data were collected for the crystals and the final structures modelled and validated.

Five mutants of XplA-heme were generated, and their activity assayed. The active site and heme environment of XplA-heme were characterized (Figure 1.2). The results revealed a possible access channel for ligand transport and, in combination with the mutational studies, demonstrated a highly unusual active site environment for substrate binding, suggesting substrate binding or catalytic roles for Gln-438 and Ala-395. The implications for the mode of action of denitration of RDX by XplA were discussed (Sabbadin *et al.*, 2009).



**Figure 1.2**

**Stereoview of the environment surrounding the heme in XplA-heme** (from Sabbadin *et al.*, 2009). The ceiling of the heme binding site is notably hydrophobic and features an unusual cluster of methionine residues, Met-318, Met-322 and Met-394, the latter held over the heme with the tip of the side chain at a distance of 8.2 Å from the iron. Ligand electron density observed at the distal face of the heme revealed the presence of the substrate analogue, imidazole. H-bonding interactions between the imidazole ligand and the water molecule (shown as a red ball) that occupies the cavity between the peptidic N-H of Ala-395 and the peptidic carbonyl group of Val-391 are shown as dashed black lines. The side chain of Gln-438, hanging over the heme and at a distance of ~3.5 Å from the imidazole ligand, can also be seen.

## Task 2

### Identification of TNT active GTases

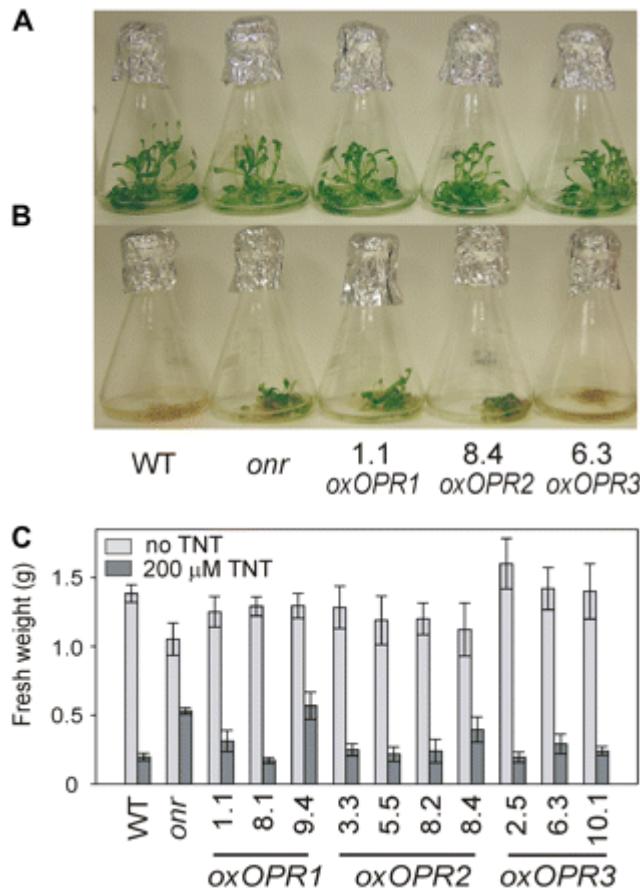
#### 2.1 Microarray experiments

To discover more about the fate of TNT in plants, and investigate how plants detoxify this compound, we used a microarray approach to identify genes involved in the detoxification process. Detailed materials and methods are described in Gandia-Herrero *et al.*, (2008). Arabidopsis seedlings (ecotype Col-0) were grown in liquid culture and exposed to 60 µM TNT for 6 hr. Microarray analysis was performed using pair-wise replicates, comparing 14-day-old seedlings treated with TNT for 6 hr with seedlings treated with solvent (DMF) only. We identified several candidate gene families that had increased transcript levels (14-173-fold) in response to TNT treatment: UDP-glycosyltransferases (GTases), oxophytodienoate reductases (OPRs), glutathione-S transferases (GSTs) and cytochromes P450. Detailed characterization of six GTases upregulated by TNT revealed that nitroreduced TNT transformation products are conjugated to sugars as part of the detoxification process in

plants. In addition, plants over-expressing GTases produced increased levels of TNT glucosides and had increased root lengths, when grown on agar plates containing TNT. This work has been published in The Plant Journal (Gandia-Herrero *et al.*, 2008).

## 2.2 Oxophytodienoate reductases (OPRs)

Studies on the OPRs revealed their role in the detoxification process. The OPRs are a small family of five or six nitroreductases, that share strong identity (43 %, 44% and 36 % for OPR1, 2 and 3, respectively) with the microbial TNT detoxifying enzyme pentaerythritol tetranitrate reductase (PETNr). Our studies of PETNr demonstrated that its expression in tobacco plants confers resistance to, and the ability to detoxify, TNT. These experiments have been described in detail in French *et al.*, (1999). Upregulation of the Arabidopsis OPRs in our microarray data implies a role in the plant's endogenous TNT detoxification system. Thus, we analysed the Arabidopsis OPRs, to assess their ability to detoxify TNT. Our experiments demonstrated that the three predominantly expressed forms, OPR1, OPR2 and OPR3, were able to transform TNT to hydroxylaminodinitrotoluene (HADNT) and aminodinitrotoluene (ADNT) intermediates (Figure 1.1). In addition, OPR1 was able to catalyse the production of hydride and dihydride Meisenheimer complexes, an activity previously observed in PETNr.



**Figure 2.1 Morphology and biomass of overexpressing OPR (oxOPR) lines treated with TNT.** A, Three-week-old, liquid culture-grown Arabidopsis, growing without TNT. B, Appearance 7 d after dosing with 200  $\mu$ M TNT. C, Fresh weight biomass after 2 weeks. Results are means of four to five biological replicates  $\pm$  standard error. (*onr*, PETNr-expressing line; WT, wild-type.)

Over-expression of OPR1 in *Arabidopsis* conferred an increased ability to take up and transform TNT from liquid media, and maintain increased biomass, compared with unmodified plants, in the presence of TNT. The opposite effect was seen in OPR1,2 RNAi knockdown lines, which took up less TNT from liquid culture and produced fewer transformation products than wild-type. OPR1 and OPR2 over-expressing lines also exhibited enhanced tolerance to TNT when grown on solid media, with seedling root lengths significantly longer than wild-type (Figure 2.1). We concluded that, along with additional detoxification mechanisms, OPRs have a physiological role in TNT detoxification. Details of these experiments may be found in our Plant Physiology paper (Beynon *et al.*, 2009).

### 2.3 Glutathione-S-transferases (GSTs)

As mentioned above, several glutathione-S-transferases (GSTs) were also up-regulated in the microarray data. The GSTs are a medium-sized family of 48 members, which can be categorized into eight groups, based on structural and catalytic features. The eight GSTs with the highest level of up-regulation in the microarray data all belong to the Tau class, a sub-group with characterized ability to catalyze herbicides. The GSTs most highly up-regulated by TNT were recombinantly expressed and found to have activity towards TNT, specifically via the reduction of a nitro group, and release of nitrite, potentially making the aromatic ring vulnerable to cleavage by dioxygenases and subsequent mineralization of TNT. Our work with these GSTs is described in detail under Task 5.

## Task 3

### Selection of range grasses

The flowering plant, *Arabidopsis thaliana* (*Arabidopsis*), is an important model system for identifying genes and determining their functions. Well-established *Arabidopsis* transformation and molecular biology protocols have enabled us to test our target genes rapidly. However, *Arabidopsis* is not a suitable species for *in situ* phytoremediation on training ranges. Grass plants, with growth habits enabling them to withstand disruption by fire and heavy equipment would be better suited to this purpose. For our experiments, germplasms derived from three native grass species; Western wheatgrass (*Pascopyrum smithii*), slender wheatgrass (*Elymus trachycaulus*) and Siberian wheatgrass (*Agropyron fragile*) were initially chosen, on the basis of their growth properties and ability to tolerate difficult soil conditions. Western wheatgrass is often used in seed mixtures for range seeding and re-vegetation of saline and alkaline areas. It is an excellent erosion control plant because of its strong, spreading rhizomes. It is adapted to a variety of soils and growing conditions, especially those found in the west and midwest regions of the USA (USDA Plant Fact Sheet). However, western wheatgrass performs poorly in the East and is not recommended for any use in this region.

Slender wheatgrass is a fast-growing, though relatively short-lived plant, recommended for inclusion in reclamation mixes because of its good seedling vigour and establishment qualities. It is widely distributed in the USA, especially in the north east, north and west regions, where it grows at elevations from 4500 ft to 12000 ft. It has good tolerance of different soil conditions, though is less drought tolerant than western wheatgrass (USDA Plant Fact sheet).

Siberian wheatgrass is a type of crested wheatgrass. It is a long-lived, cool season perennial grass, commonly found in the western United States. It is ideal for land reclamation in areas with only 8 - 20 inches' annual precipitation, due to its drought tolerance, good seedling vigour, extensive fibrous root system and resistance to fire (USDA Plant Fact Sheet).

Because of the properties listed above, these wheatgrass plants would be highly suitable for engineering with transgenes designed to detoxify RDX and TNT in the soil environment. Our experiments to engineer transgenic wheatgrass are described in detail in Task 6.

At a later stage in the project (2012), our attention turned to switchgrass (*Panicum virgatum* L) because increasing interest in this species as a biofuel crop has recently led to the development and publication of efficient transformation techniques. Switchgrass is climatically adapted to grow throughout most of the USA, except California and the Pacific North West. It is tolerant of a wide range of soil types and can stabilise soil through its strong rhizomatous growth. It also has potential as a biofuel crop. Our experiments to engineer switchgrass for phytoremediation purposes are described in Task 6.

## **Task 4**

### **Development of methods for transformation of grasses**

At the start of this project, there was no established methodology for the transformation of wheatgrass species. We therefore had to develop a transformation system to engineer wheatgrasses to express our target genes. As a platform for this, we used creeping bentgrass (*Agrostis stolonifera*); a model, perennial grass species, with a well-developed transformation protocol, to produce transgenic bentgrass lines expressing XplA and NR. We hoped to transfer this technology to the range grasses described in Task 3.

Detailed descriptions of the experiments that were conducted with western wheatgrass and creeping bentgrass are provided under Task 6.

In the early stages of the project (completed March 2008), significant progress was made towards engineering western and slender wheatgrasses for RDX phytoremediation. Tissue culture methods were optimized for the reliable generation of embryogenic callus suitable for biolistic transformation, and the subsequent regeneration of transgenic western wheatgrass lines from callus cultures was established. However, the ratio of regeneration from embryogenic callus was low, which was an obstacle for successful transformation of this species. The transformation and regeneration efficiencies for western wheatgrass were improved as the project progressed. Focus was transferred to switchgrass at later stages of the project, so that by May 2015, switchgrass plants engineered to express functional XplA/B and NR proteins had been generated and were ready for planting in demonstration-scale field trials (Task 11).

## **Task 5**

### **Multiple transgenics:**

### **Expression of NR, XplA or XplA variants and GTases in *Arabidopsis*.**

#### **5.1 Expression of XplA/B and NR in *Arabidopsis***

The research described in this section builds on work that was carried out for a previous SERDP report (ER-1318). The model plant system, *Arabidopsis*, was used in these experiments, for ease of genetic manipulation.

*Arabidopsis* plants were engineered to express the transgenes *xplA* and *xplB*, for RDX degradation. In order to remove RDX from the environment, plants need to be able to tolerate the toxicity of TNT, which often occurs as a co-contaminant. Hence, the *Arabidopsis* plants were also engineered to express the *nfsI* gene for nitroreductase (NR), for detoxification of TNT. Liquid culture studies were performed and soil experiments were carried out, to observe the effects of RDX and TNT on the transgenic plants and to assess their effectiveness at removing these explosives from soil leachate. This would provide an indication of the potential suitability of a transgenic plant system for phytoremediation applications.

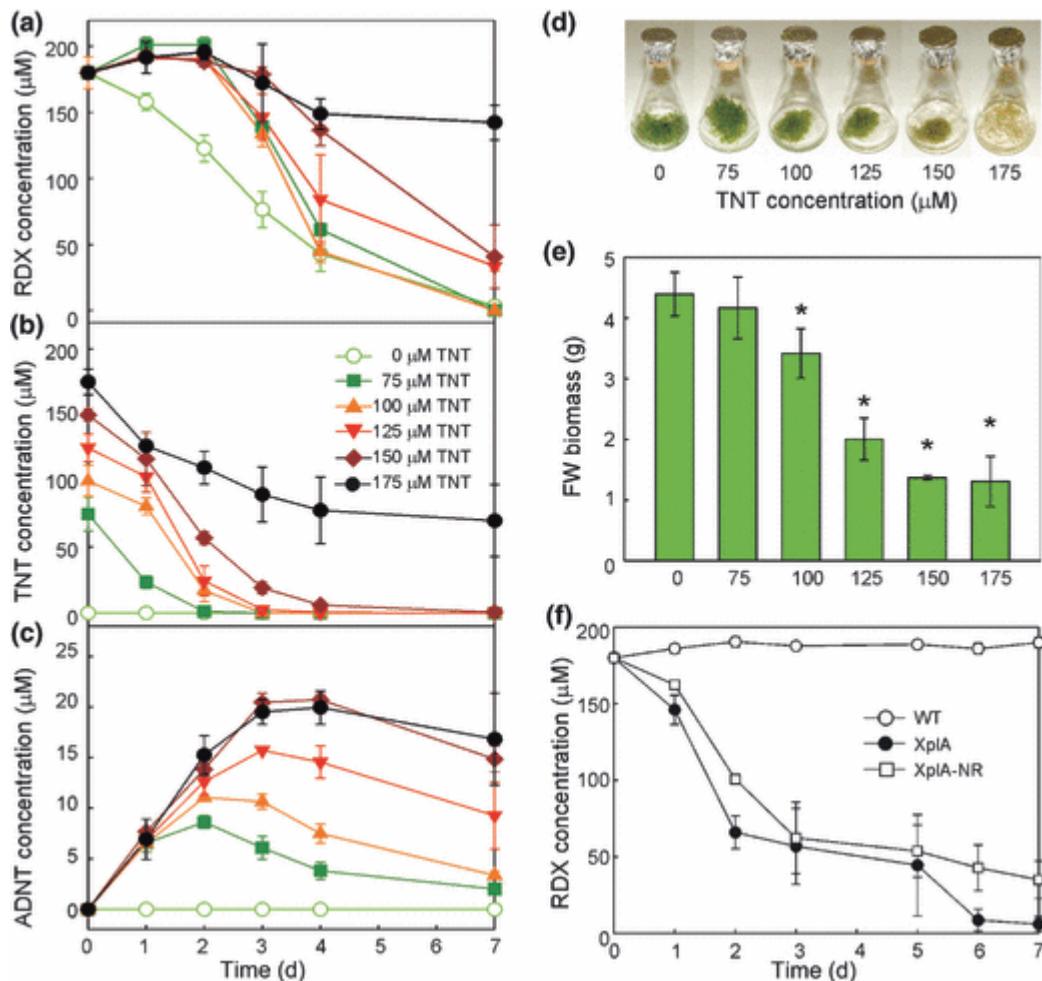
The experiments were published in Rylott *et al.*, (2011b).

### 5.1.1 Production of transgenic plant lines

The *xplA*, *xplB* and *nfsI* genes were cloned and introduced into Arabidopsis (Col-0) plants under the control of the *CaMV35S* promoter and *ocs* terminator. Homozygous transgenic plant lines were created, containing *xplA*, *xplA* and *nfsI* (XplA-NR), *xplA* and *xplB* (XplA-XplB), as well as the triple transgenic (XplA-XplB-NR). Details of the experimental procedures and plant lines produced are presented in the Appendix (A5.1). Transgene expression analysis was carried out using qRT-PCR and Western blots verified production of the relevant proteins (Appendix A5.2).

### 5.1.2 Liquid culture experiments

To test whether the XplA-NR plants could remove both RDX and TNT from liquid culture medium, liquid culture experiments were performed, as described previously (Rylott *et al.*, 2006; Rylott *et al.*, 2011b). Seedlings were initially grown on agar plates containing  $\frac{1}{2}$ MS medium, and were then transferred to flasks containing liquid  $\frac{1}{2}$ MS medium amended with 20 mM sucrose and dosed with 180  $\mu$ M RDX and a range of TNT concentrations (75, 100, 125, 150 and 175  $\mu$ M). The rate of RDX uptake decreased with increasing TNT concentration. In fact, RDX was only taken up following the depletion of TNT to below 60  $\mu$ M in the medium (Figure 5.1).



**Figure 5.1 Uptake of RDX and TNT by XplA-NR-expressing Arabidopsis plants grown in liquid culture.** Levels of (a) RDX, (b) TNT and (c) amino dinitrotoluene (ADNT) in liquid media containing 10-day-old XplA-NR-expressing plants dosed with 180  $\mu$ M RDX and a range of TNT concentrations. (d) Appearance and (e) fresh weight (FW) of XplA-NR plants 7 days after dosing. (f) Levels of RDX in liquid media containing 10-day-old WT, XplA- and

XplA-NR-expressing plants dosed with 180  $\mu\text{M}$  RDX only. WT, wild-type; NR, nitroreductase. Results are the mean  $\pm$  SE of five replicate flasks with 200 seedlings per flask.  
\*Significantly different ( $p < 0.05$ ) from untreated plants.

During the course of the experiment, the photosynthetic tissues of the XplA-NR plants exhibited increased yellowing, followed by bleaching and biomass reductions that correlated with increasing TNT concentration (Figure 5.1). Despite this inhibition, the XplA-NR plants were significantly more tolerant to TNT than either the XplA-only expressing plants or the wild-type, untransformed plants, both of which were killed by TNT concentrations of 175  $\mu\text{M}$  and above (results not shown). Figure 5.1F shows that the XplA-NR plants retained the capacity to remove RDX from liquid culture, in the absence of TNT, at a rate similar to that of the parental XplA-expressing line.

When the XplA-XplB-NR lines were grown in liquid culture treated with RDX and TNT together, the plants removed all the RDX from the medium within 3 days. The parental XplA-XplB line removed only approximately 65% of the RDX in this time (Figure 5.2). The XplA-XplB-NR lines also removed TNT more quickly than the XplA-XplB lines.

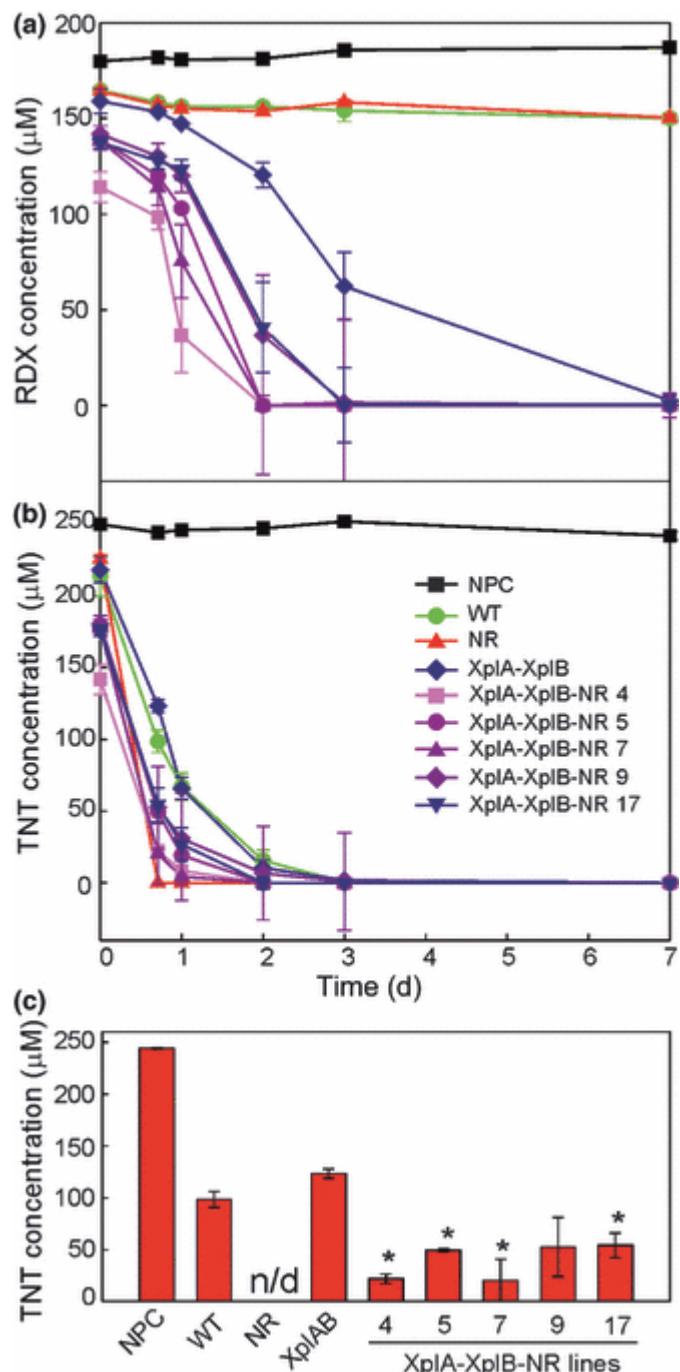
### 5.1.3 Soil experiments

Experiments were carried out to monitor the tolerance of XplA-NR plants to TNT contamination in soil.

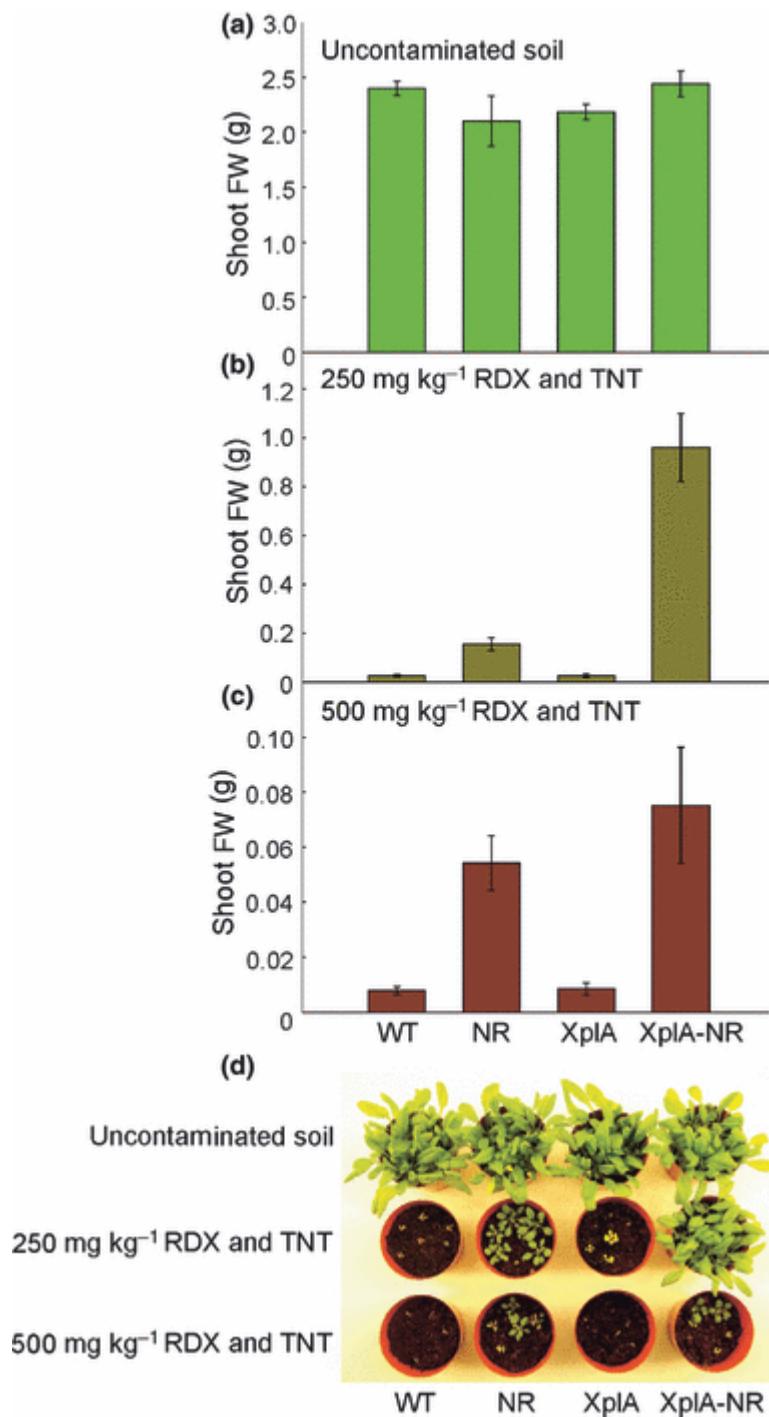
Plants were grown in soil treated with either 250 or 500  $\text{mg kg}^{-1}$  of RDX and TNT, for 6 weeks. In untreated soil, the appearance and biomass of the XplA-NR plants were similar to those of the XplA and NR plants (Figure 5.3). In the presence of RDX and TNT, the growth of all the plants was reduced significantly. As expected, the NR-expressing plants had higher shoot biomasses than wild-type plants (Figure 5.3). However, the XplA-NR plants had the highest shoot biomasses, especially at the 250  $\text{mg kg}^{-1}$  concentration.

To test whether the XplA-XplB-NR lines were able to remove RDX migrating through the soil column, soil leachate levels of plants watered with RDX were measured, using the methods described previously (Jackson *et al.*, 2007) (Figure 5.4).

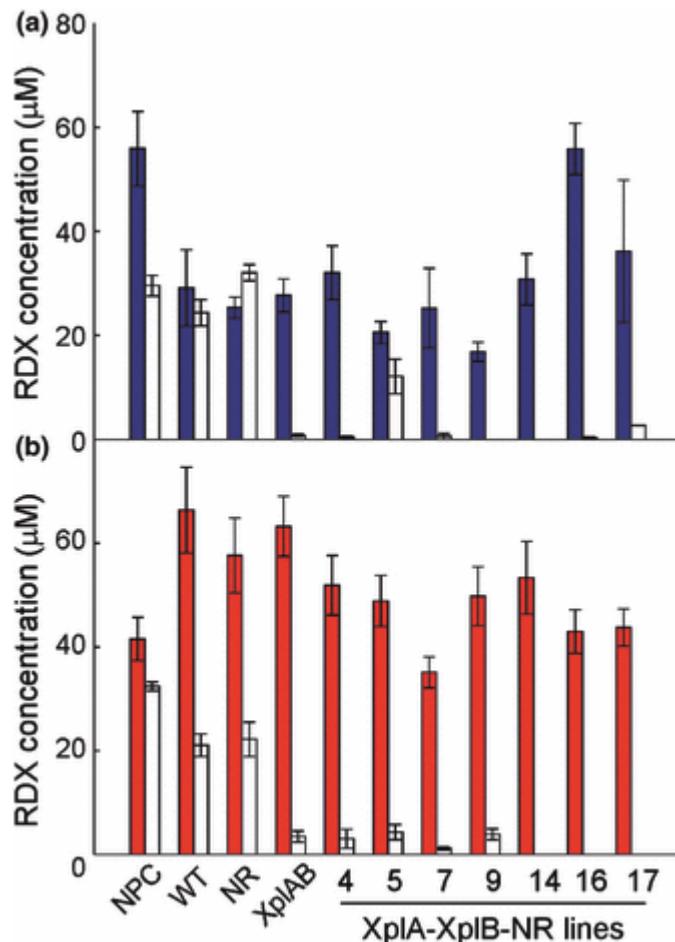
The reduced RDX levels observed in the 'no plant' controls can be attributed to differences in soil properties in the absence of plant roots, enabling liquid to travel more quickly through the soil column. All the XplA-XplB-NR lines, (except line 5), removed RDX from soil leachate at rates comparable with the XplA-XplB parental line. The concentration of RDX in the soil leachate after 7 days from pots in which XplA-XplB-NR and XplA-XplB lines were growing was significantly less ( $p < 0.001$ ) than the level of RDX in leachate from pots in which wild-type plants were growing. To test whether the XplA-XplB-NR lines were still able to remove RDX from soil leachate in the presence of TNT, the soil leachate experiment was repeated using plants grown in soil treated with 100  $\text{mg kg}^{-1}$  TNT (Figure 5.4). Seven days after dosing, the level of RDX in the soil leachate from pots containing the XplA-XplB-NR lines was significantly less ( $p < 0.001$ ) than the level of RDX in soil leachate from wild-type plants. Measurements on the aerial parts of the plants showed that levels of RDX in the XplA-XplB and XplA-XplB-NR plant shoots were much lower than those in the wild-type and NR-only plants, when grown on soil treated with 100  $\text{mg kg}^{-1}$  RDX and TNT (Rylott *et al.*, 2011b)



**Figure 5.2 Uptake of RDX and TNT by XplA-XplB-NR-expressing *Arabidopsis* plants grown in liquid culture.** Plants were dosed with 180  $\mu\text{M}$  RDX and 250  $\mu\text{M}$  TNT. Levels of (a) RDX and (b) TNT in the culture medium. (c) Levels of TNT in the medium 17 hr after dosing. NPC, no plant control; WT, wild-type; NR, nitroreductase; n/d, not detected. Results are the mean  $\pm$  SE of four replicate flasks. \*Significantly different ( $p < 0.05$ ) from value for XplA-XplB plants. (From Rylott *et al.*, 2011b).



**Figure 5.3 Growth of XplA-NR-expressing *Arabidopsis* plants in soil treated with RDX and TNT.** Shoot fresh weights (FW) of 6-week-old plants grown in (a) uncontaminated soil, (b) 250 mg kg<sup>-1</sup> RDX and TNT and (c) 500 mg kg<sup>-1</sup> RDX and TNT. (d) Appearance of plants after 6 weeks' growth in soil treated with both RDX and TNT. WT, wild-type; NR, nitroreductase. Results are the mean  $\pm$  SE of five replicate pots.



**Figure 5.4 Levels of RDX in soil leachate from 6-week-old *Arabidopsis* plants watered with 180  $\mu\text{M}$  RDX.** Leachate was collected following watering ( $t = 0$ , closed bars), and then plants were flushed with an equal volume of water after 7 days ( $t = 7$ , open bars). Plants were grown in (a) untreated soil or (b) soil treated with 100  $\text{mg kg}^{-1}$  TNT. NPC, no plant control; WT, wild-type; NR, nitroreductase. For all groups, the results are the mean  $\pm$  SE of eight replicate pots (from Rylott *et al.*, 2011b).

#### 5.1.4 Summary

In liquid culture, the uptake of RDX by XplA-NR and XplA-XplB-NR plants was reduced in the presence of TNT. However, once the transgenic lines had removed the TNT, they were able to take up and degrade the RDX. The XplA-XplB-NR lines were able to remove RDX more quickly from the liquid culture than the other lines. Following uptake, TNT is localized almost entirely within the root tissues, whereas RDX is translocated to the aerial parts of the plant (Vila *et al.*, 2007). Thus, in liquid culture, where the aerial parts of the submerged plant are exposed to TNT, inhibition of RDX degradation by XplA-expressing plants is not unexpected. Given that the growth of plants in liquid culture is far-removed from more natural growth conditions, soil-based experiments were carried out, to measure the removal of RDX by plants from soil contaminated with TNT. The XplA-XplB-NR plants were able to remove significantly more RDX from soil leachate than either wild-type or NR-only plants. Also, XplA-NR plants grown in soil contaminated with both RDX and TNT had higher biomasses than plants expressing NR alone, indicating that RDX can be utilized as a nitrogen source by XplA-expressing plants.

Taken together, these results show that our transgenic *Arabidopsis* plants can withstand TNT toxicity and remove RDX from soil water. The results suggest that RDX can then be broken

down within the plants into less harmful nitrogenous compounds, which can in turn be used as a growth substrate for the plants. This means that the RDX is removed completely from the soil environment, indicating that a transgenic plant system could have excellent potential for successful phytoremediation of land contaminated with explosives.

## 5.2 The role of monodehydroascorbate reductase 6 (MDHAR6) in TNT toxicity

### 5.2.1 Introduction

To identify additional genes conferring enhanced tolerance to TNT a forward genetic screen of mutated *Arabidopsis* seed lines was carried out on media containing TNT, and a TNT-resistant, recessive mutant was isolated. The mutation was mapped to *MONODEHYDROASCORBATE REDUCTASE 6 (MDHAR6)*, known formerly as *MDAR6*.

MDHARs are enzymes that play roles in the antioxidant systems of plants and are involved in multiple stress responses, such as desiccation tolerance (Lopez-Cristoffanini *et al.*, 2015). They are oxidoreductases that utilize NAD(P)H as an electron donor to reduce FAD, which then reduces monodehydroascorbate (MDA), the free-radical oxidation product of ascorbate (Hossain & Asada, 1985). This has been demonstrated using purified cytosolic MDHAR from *Cucumis sativus* (cucumber) and *Spinacia oleracea* (spinach) (Sano *et al.*, 1995; Sano *et al.*, 2005). MDHAR also functions in other antioxidant pathways; for example, it has been shown to reduce phenoxyl radicals to their respective parent phenols, via a mechanism similar to the reduction of MDA (Sakihama *et al.*, 2000; Sakihama *et al.*, 2002). As yet, the activity of *Arabidopsis* MDHAR6 towards its proposed substrate MDA has not been demonstrated, but as indicated above, this enzyme might have alternative endogenous roles.

The *Arabidopsis* genome contains five *MDHAR* genes. Microarray expression profiling shows that all 5 *MDHARs* are expressed at significant levels both temporally and spatially throughout the plant, with *MDHAR6* expressed predominantly in plant roots, the site of TNT detoxification. *MDHAR1* (Lisenbee *et al.*, 2005; Leterrier *et al.*, 2005) and *MDHAR4* (Eastmond, 2007) are targeted to the peroxisomal matrix and membrane, respectively, while *MDHAR2* and *MDHAR3* are cytosolic.

In *Arabidopsis*, *MDHAR6* contains four splice variants, encoding transcripts that have been shown to target protein to the chloroplast (*MDHAR6.1,3* and *4*) and the mitochondrion (*MDHAR6.2*). *MDHAR6.2* includes seven extra N-terminal codons, required for targeting to the mitochondria (Obara *et al.*, 2002).

Consistent with its role in stress response pathways, studies have shown that over-expression in *Arabidopsis* of a chloroplast-targeted MDHAR from tomato confers enhanced resistance to temperature and methyl viologen (Paraquat) (Li *et al.*, 2010). Overexpression of *Arabidopsis* *MDHAR1* in tobacco cytosol confers tolerance to ozone, salt and polyethylene glycol stresses (Eltayeb *et al.*, 2007).

To understand more about the role of *Arabidopsis* *MDHAR6* in TNT tolerance, various experiments were undertaken. *mdhar6* mutants were obtained and complementation studies and phenotypic characterisation were carried out. *MDHAR6* was expressed in *E.coli* and activity assays were conducted. The results have been published in *Science* (Johnston *et al.*, 2015).

### 5.2.2 Experiments with *mdhar6* mutants

#### 5.2.2.1 Plant material

Wild-type *Arabidopsis* ecotypes Columbia (Col-0), Col-7 and Nossen (Nos) were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC) (University of Nottingham, UK).

To find genes directly involved in TNT detoxification, a CaMV 35S activation library was obtained from NASC (Weigel *et al.*, 2000). 7,200 activation-tagged lines were screened on 7µM TNT, a concentration shown to inhibit root growth in wild-type seedlings (Gandia-

Herrero *et al.*, 2008). A mutant with enhanced root growth was isolated and mapped to *MDHAR6*, as described in Eastmond (2007). A thymine deletion was identified, 2297 bp from the ATG start codon in exon 11 of the genomic sequence of *MDHAR6*. This mutation (*mdhar6-1*) is predicted to result in a frame shift and the introduction of an early stop codon, causing a truncation of more than a third of the protein (Figure 5.5).

The *mdhar6-2* mutant, in the Nos background, was obtained from the RIKEN Arabidopsis transposon-tagged mutant (RATM) lines (Ito *et al.*, 2005) from RIKEN BioResource Center (Japan). Sequencing of the region flanking the transposon (using primers MDAR6-1227 5'-TTCCCTGCGTCTGTGGTGAG-3' and Ds3-4 5'-CCGTCCCCAAGTTAAATATG-3') confirmed the insertion to be inserted 654 bp from the ATG, between exon 3 & 4.

The *mdhar6-3* mutant, in the Col-0 background, was obtained from the GABI-Kat T-DNA mutagenized lines (line ID 258H07) (Kleinboelting *et al.*, 2012). Sequencing of the region flanking the border region of the T-DNA (using primers MDAR6-514 5'-CTTCTCCACCACCTCCAAC-3' and o8409 5'-ATATTGACCATCATACTCATTGC-3') confirmed a T-DNA insert 76 bp upstream of the ATG start codon in the genomic sequence of *MDHAR6*.

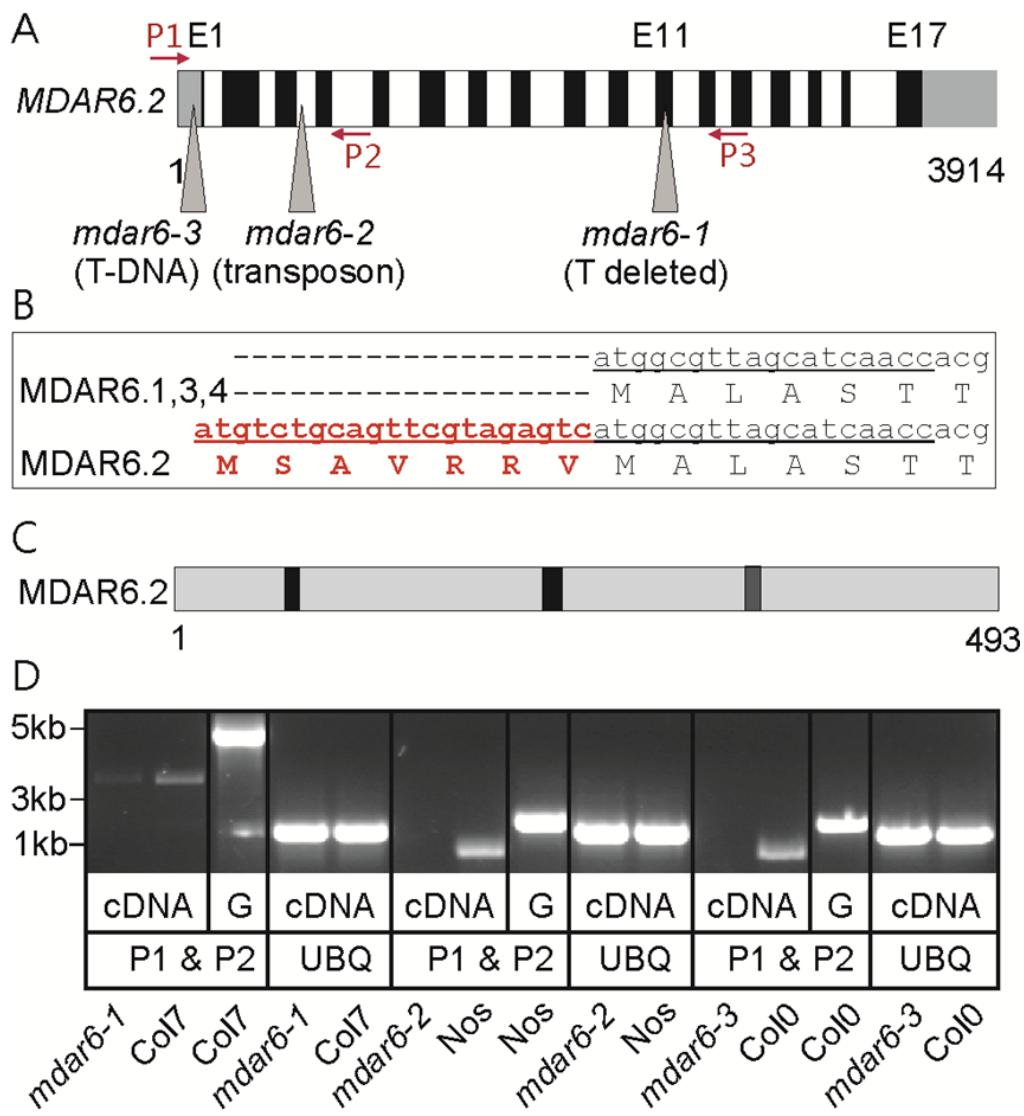
### 5.2.2.2 Characterisation and complementation of *mdhar6-1*

Because the *mdhar6-1* phenotype is recessive, suggesting a loss-of-function mutation, complementation studies were carried out. A wild-type copy of *MDHAR6* was introduced into the *mdhar6-1* mutant. To do this, PCR was used to amplify the mitochondrial-targeted form of *MDHAR6* (*MDHAR6.2*). The sequence, under the control of the near-constitutive 35S promoter, was cloned into a binary vector and introduced into Col-7 and *mdhar6-1* plants, using *Agrobacterium*-mediated floral dipping. (See Appendix A5.3 for details).

The phenotypes of the wild-type, mutant and transgenic lines were characterised. Seeds were surface-sterilised, then germinated on agar plates containing ½MS (Murashige & Skoog, 1962), amended with 20mM sucrose and 7µM TNT. Seedlings were grown for 7 days and their root lengths measured. The results (Figure 5.6) showed that *mdhar6-1* seedlings had longer roots than Col-7 (wild-type) when grown on medium containing TNT. In *mdhar6-1* lines transformed with 35S *MDHAR6.2*, this long root phenotype was fully restored to wild-type. Col-7 lines over-expressing *MDHAR6.2* showed no further decrease in root length, relative to wild-type controls, in the presence of TNT.

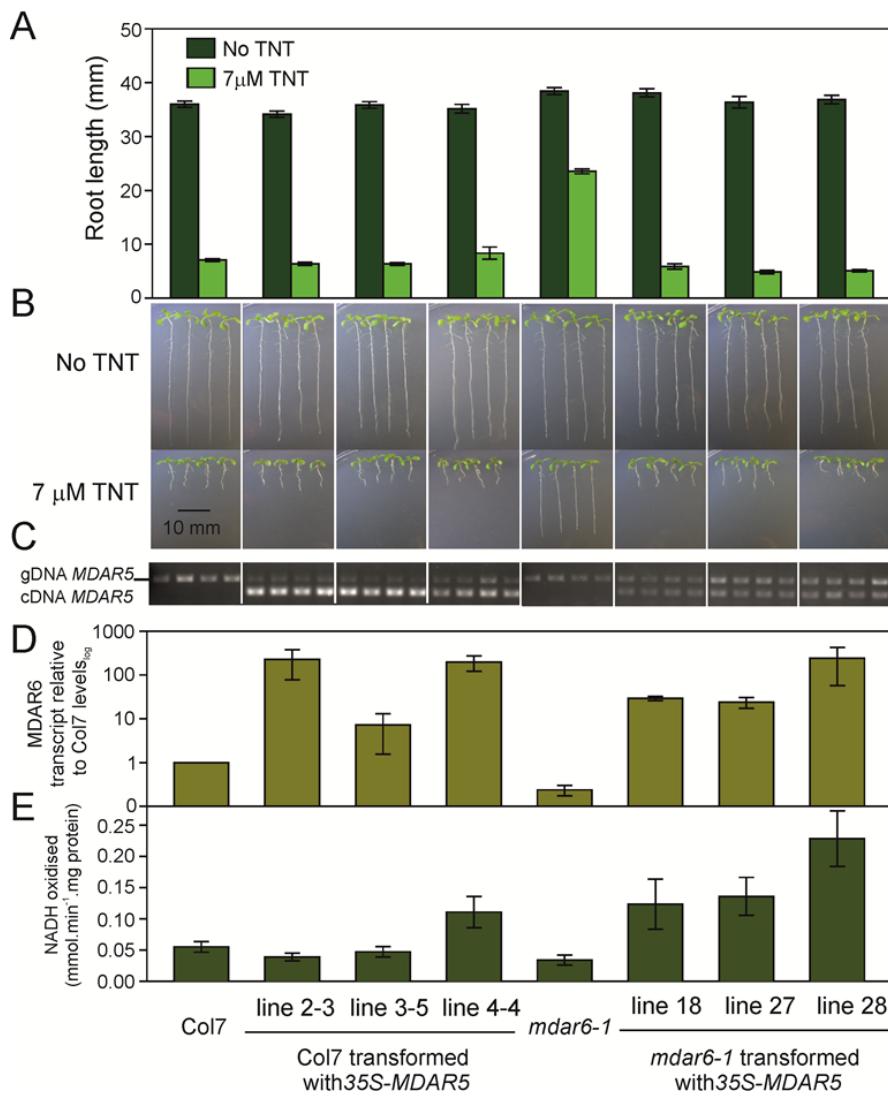
Transcript levels of *MDHAR6* were measured using qRT-PCR, as described in Rylott *et al.* (2011b). *mdhar6-1* plants had approximately 30% less *MDHAR6* transcript than wild-type Col-7. In the Col-7 and *mdhar6-1* lines transformed with 35S *MDHAR6.2*, mean transcript levels from three independent lines were much higher than in wild-type Col-7 (figure 5.6). *MDHAR6* transcript was disrupted by DNA insertions in *mdhar6-2* and *mdhar6-3* (Figure 5.5).

To assess the impact of altered transcript levels on MDHAR activity, a spectrophotometric assay following oxidation of NADH was used to determine MDHAR activity in crude extracts from rosette leaves, as described previously (Colville & Smirnoff, 2008). *mdhar6-1* plants had approximately 40% less MDHAR activity than wild-type (Figure 5.6). However, in the 35S-*MDHAR6.2* lines (Col-7 background), only moderate increases in activity were observed, compared with transcript levels, suggesting that MDHAR6 expression is controlled post-translationally. Interestingly, the level of activity in the *mdhar6-1* 35S-*MDHAR6.2* lines was 2.9-fold higher than that of wild-type, suggesting that post-translational regulation is aberrant in the *mdhar6-1* background.



**Figure 5.5 To-scale representation of mutations in *MDHAR6*.**

A) Genomic *MDHAR6* showing *exons* (*E*) as black boxes, 5' and 3' *UTRs* as grey boxes. Locations of *mdhar6-1*, *mdhar6-2* and *mdhar6-3* disruptions are shown as grey arrows. Position of primers (P1, P2 and P3) used in PCR analysis shown as arrows. Numbers refer to base pairs from start of genomic sequence. B) 5' region of cDNA and amino acid sequences showing chloroplast transit peptide sequences (underlined) present on MDHAR6.1,3,4 and mitochondrial transit peptide sequence (Bold) present on MDHAR6.2. C) Encoded MDHAR6. Black boxes show location of motifs thought to be involved in binding ADP in NAD and FAD. Dark grey box highlights region thought to be involved in binding FMN. Numbers refer to amino acids from start methionine. D) RT-PCR analysis on cDNA and genomic (G) sequences of *mdhar6-1*, *mdhar6-2*, *mdhar6-3* and wild-type control lines. Primers used as shown in A) above, along with ubiquitin (UBQ) control primers.



**Figure 5.6 Complementation of *mdhar6-1*.**

The *mdhar6-1* mutant was transformed with 35S-MDHAR6.2 cDNA. (A) Root lengths and (B) appearance of seven-day-old seedlings with and without 7  $\mu$ M TNT. Results are means  $\pm$  SE of 30 seedlings from each of three plates. (C) PCR on DNA from seedlings using intron-spanning primers specific for *MDHAR6*. (D) Expression of *MDHAR6* transcript levels in rosette leaves, normalised to actin and relative to Col-7 levels. Results are means  $\pm$  SE of cDNA made from RNA extracted from five replicate plants. (E) MDHAR activity in rosette leaves. Means  $\pm$  SE of crude extracts from five replicate plants.

Seedlings of both *mdhar6-2* and *mdhar6-3* mutants had longer roots than wild-types, when grown on all concentrations of TNT tested, although *mdhar6-3* seedlings were less resistant to TNT than *mdhar6-1* and *mdhar6-2* (Figure 5.7). Enzyme activity assays showed that the *mdhar6-2* plants had approximately 45% less MDHAR activity than Nos wild-type. However, levels of MDHAR in *mdhar6-3* plants were not significantly different from wild-type (Figure 5.7).

Liquid culture studies were carried out to measure rates of TNT uptake from liquid medium. Three-week-old plants grown in liquid culture were dosed with 20 mM sucrose containing

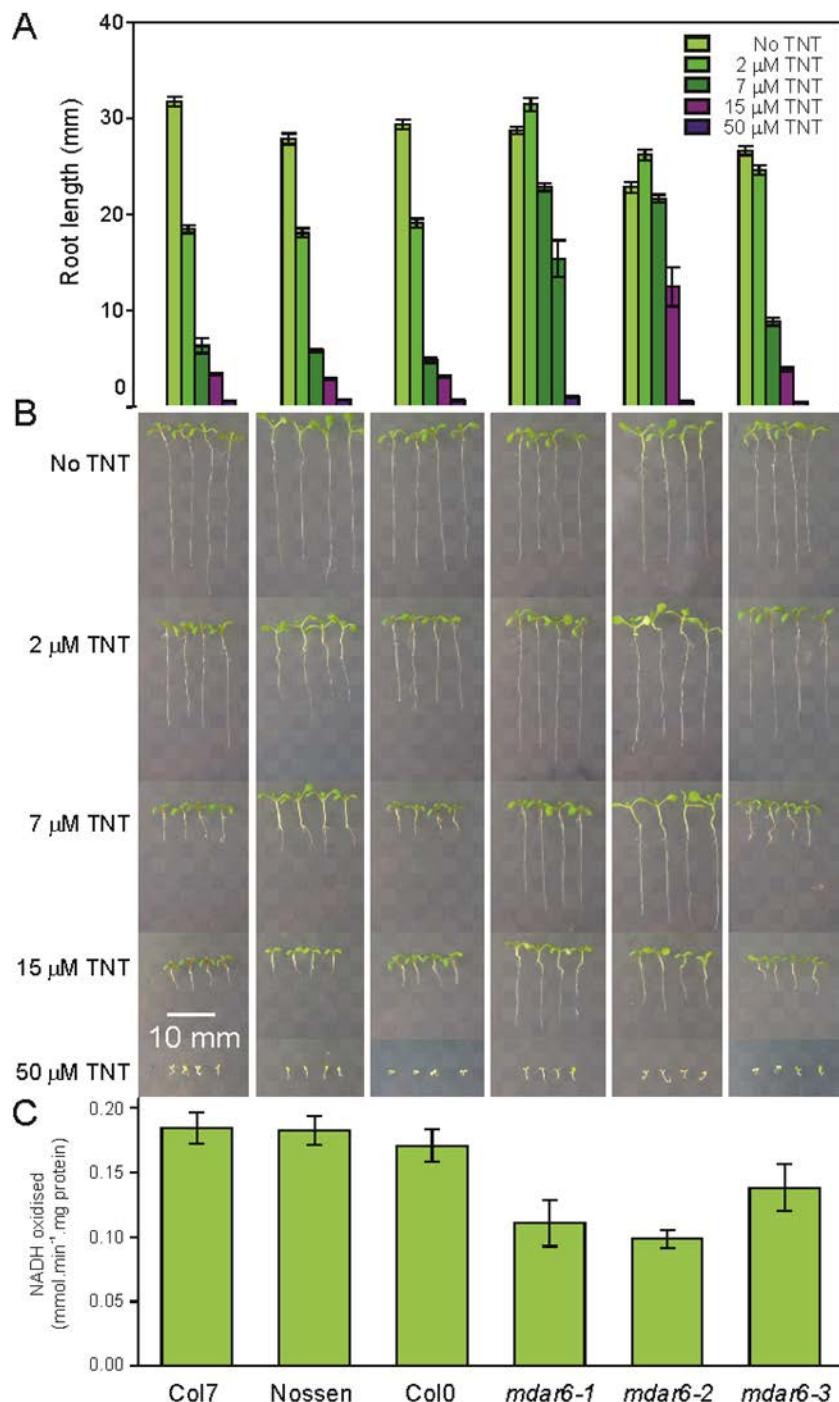
100 or 200  $\mu$ M TNT and the rate of TNT uptake followed. The growth, appearance and TNT uptake rates of all three *mdhar6* lines were not significantly different from wild-type (results not shown).

To examine whether the *mdhar6* lines exhibited enhanced resistance to TNT beyond the seedling stage, five-day-old seedlings were planted into soil treated with TNT at 0, 50, 100, 150, 200 and 300 mg/kg soil, and grown for six weeks, as described in Rylott *et al.*, (2011b). Consistent with the root length studies, the aerial parts and the roots of *mdhar6* plants had increased biomasses compared with wild-type controls. These differences were apparent soon after transfer to TNT-treated soil, until the end of the six week experiment. Additionally, *mdhar6-1* lines complemented with 35S-*MDAR6.2* showed tolerances to TNT that were comparable to wild-type (Col-7), supporting the hypothesis that the TNT-resistant phenotype observed in seedlings was also due to disruption of MDHAR6 in mature plants.

Apart from the increased biomass in the presence of TNT, no differences in phenotype were observed during seedling development or (in soil-grown plants) throughout the life-cycle. Despite studies showing that up-regulation of MDHAR activity can increase resistance to a range of abiotic stresses, no increased resistance was seen when the *mdhar6* lines were tested on paraquat, hydrogen peroxide, salt or sorbitol, suggesting that the tolerance to TNT is not due to enhanced general defences.

Previous work on TNT phytoremediation has focused on improving TNT metabolism, so that remediation can occur more quickly. However, enhanced TNT tolerance could still allow *mdhar6* plants to remove more TNT from the soil than wild-type plants, through enhanced biomass. A greater network of roots would also provide more dispersed contact with the soil, which could enhance the potential for remediation.

The *mdhar6* complementation studies described above raised additional questions. For example, although full complementation was observed, why did the 35S-*MDAR6.2* over-expression lines not exhibit enhanced sensitivity to TNT? Also, is chloroplastic MDHAR6 involved in TNT detoxification? To address these questions, the plastid-targeted splice variant, *MDHAR6.1*, was cloned into the *Kpn*I and *Bam*HI sites of *pART7* and the 35S-*MDHAR6.1-nos* cassette transferred into *pART27*. This vector was introduced into *Agrobacterium tumefaciens* GV3101, and *mdhar6-1* and Col7 background ecotype lines were transformed. Transformants were selected on 7 $\mu$ M TNT. PCR was performed using intron-spanning primers, to confirm the presence of the 35S-*MDHAR6.1* transgene in these lines. The root lengths of the homozygous lines were measured. It was found that the plastid-targeted MDHAR6.1 partially restored TNT toxicity (Johnston *et al.*, 2015), but did not fully complement the TNT-resistant phenotype, as the mitochondrial MDHAR6.2 had done.



containing TNT.  
and without 7 µM TNT.  
(C) MDHAR activity in  
rude extracts from five

### 5.2.2.3 Recombinant expression of MDHAR6 in *E.coli*.

The *MDHAR6.1* transcript was amplified from cDNA derived from 14-day old Arabidopsis seedlings using PCR. The PCR product was cloned into *pET16b* and its sequence verified. The plasmid was used to transform aliquots of *E.coli* competent cells and the MDHAR protein was expressed and purified. Details of these procedures are described in the Appendix (A5.4.2).

MDHAR activity was then determined using a spectrophotometric assay following oxidation of NADH, according to a protocol modified from Colville & Smirnoff (2008) (see Appendix; A5.4.3).

It was found that most of the MDHAR protein expressed in this way was insoluble. Hence, in an attempt to improve solubility, *MDHAR6* was re-cloned into *pGEX-4T3* which has an N-terminal GST tag that can improve solubility. The chloroplast-targeted splice variant, *MDAR6.1*, was PCR-amplified using primers which included *Bam*H I and *Sal*I restriction enzyme sites, and cloned, via a sequence-confirmed, *pJET* intermediary cloning vector, into *pGEX-4T3*. This new construct, *pGEX-4T3-MDAR6.1* was then cloned into *E. coli* and the solubility of the expressed protein tested.

Using this protocol, the majority of the protein was still in the insoluble fraction. However, sufficient active protein was recovered to enable activity assays to be performed. MDHAR activity was assayed in the his-tag purified and non-purified soluble fractions. MDHAR activity was confirmed in the purified MDHAR6. Activity towards TNT was confirmed by following the oxidation of the co-factor NADH spectrophotometrically at A340nm. As a control, the same purification procedure was carried out on *E.coli* transformed with empty pET16b vector. This gave a background NADH oxidation rate of approximately 5 $\mu$ mol/min/mg protein, which remained unchanged upon addition of TNT. The purified MDHAR6 gave a background rate approximately 10-fold higher than the empty vector control. This could be due to turnover of molecular oxygen by MDHAR6, or activities of contaminating *E.coli* enzymes in the protein. (SDS-PAGE estimates of purification suggest that MDHAR6 comprised about 20% of the total protein). However, upon the addition of TNT to the purified MDHAR6, the rate of NADH oxidation doubled (to 100  $\mu$ g/min/mg protein). An additional control, testing the effect of adding DMSO (solvent) only, gave no increase in NADH oxidation. To investigate the products formed by MDHAR6 activity on TNT, stopped assays were run, using HPLC. There was no decrease in TNT concentration over the time-course of the assay. We hypothesised that MDHAR6 performs a one electron reduction of TNT to generate a TNT nitro-radical, which then undergoes spontaneous auto-oxidation back to TNT. Colleagues in the Chemistry Department at the University of York conducted electron spin resonance (ESR) studies, to identify the TNT nitro-radical.

#### 5.2.2.4 Enzyme kinetics

The reaction mixture for kinetic assays for TNT contained 10.3  $\mu$ g/ml purified MDHAR6 in 50 mM Tris 1 mM EDTA (pH 7.6), 100  $\mu$ M NADH and 15% DMSO. Michaelis-Menten parameters were calculated using Sigma Plot v12.0. The reactions for HPLC analysis contained 52  $\mu$ g/ml MDHAR6, 200  $\mu$ M NADH and 200  $\mu$ M TNT in 50 mM Tris 1 mM EDTA (pH 7.6), 15% DMSO. HPLC analysis was carried out on the samples, using a C18 column, with isocratic conditions (water: methanol 50:50, flow rate 1 ml/minute, RDX elution monitored at 230 nm). Integrations were performed using Empower software.

Kinetics analysis on MDHAR6, using TNT as substrate, gave  $K_m$  and  $V_{max}$  values of  $522 \pm 57 \mu\text{M}$  and  $0.143 \pm 0.0067 \text{ mmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ , respectively.

#### 5.2.2.5 Electron Paramagnetic Resonance Spectrometry

In collaboration with Dr Victor Chechik in the Department of Chemistry at University of York, Electron Paramagnetic Resonance (EPR) spectrometry techniques were used to measure TNT-dependent production of superoxide.

Spectra were recorded on a Bruker EMX Micro spectrometer at X band (9.86 GHz), with modulation amplitude 1G, microwave power 5 mW, scan time 80 s and time constant 80 ms.

The activity assays contained 1.5 mg/ml MDHAR6 in 50 mM KH<sub>2</sub>PO<sub>4</sub> (pH 7), 80 mM DMPO or DEPMPO, 300 µM NADH and 500 µM TNT in DMF (end DMF concentration 1 % v/v). For assays containing SOD (Sigma S8409), 2,500 U/ml SOD in 100 mM KH<sub>2</sub>PO<sub>4</sub> pH 7.5 were added to the assay to an end concentration of 50 U/ml, before addition of TNT and NADH. MDHAR6 was denatured by boiling for 5 min. Simulation parameters for DMPO-superoxide adduct: aN = 14.09 G, aβ-H = 11.33 G, aγ-H = 1.23 G, DMPO-OH aN = 14.97 G, aβ-H = 14.68 G, DEPMPO-superoxide adduct, isomer 1 (42 %): aN = 13.03 G, aβ-H = 11.85 G, aγ-H = 0.68 G, aP = 50.76 G, and DEPMPO-superoxide adduct, isomer 2 (58%): aN = 13.15 G, aβ-H = 10.29 G, aγ-H = 0.61 G, aP 49.63 G.

Using either spin trap 5,5-dimethyl-pyrroline N-oxide (DMPO) or 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO), spectra correlating with superoxide production were observed. The DMPO-superoxide spectrum was not observed in the presence of superoxide dismutase, or when TNT was omitted, or when denatured MDHAR6 was used. These results establish that superoxide is produced during the reduction of TNT by MDHAR6. As MDHAR6 is targeted exclusively to the mitochondria and plastids, it was concluded that it is the production of damaging superoxide by MDHAR6, in combination with the location of its production within sensitive organelles, that accounts for the majority of the toxicity of TNT in plants.

To establish that superoxide production from TNT *in planta* leads to oxidative stress, 3,3'-diaminobenzidine (DAB) was used to measure H<sub>2</sub>O<sub>2</sub> generation.

Seedlings were grown for seven days on agar plates with and without 15 µM TNT. The seedlings were then vacuum infiltrated in 1 mg/ml 3,3'diaminobenzidine (DAB) in 50 mM Tris-acetate (pH 5) for 30 min. The vacuum was then released, and seedlings incubated for a further 2.5 hr in the dark at room temperature. Images were taken using a Nikon SMZ800 dissection microscope with AxioVision Rel. 4.5 software.

In seedlings grown for seven days on agar plates containing 15 µM TNT, the intensity of colour (due to DAB oxidation by peroxide) was much higher in wild-type roots than in *mdhar6-1* roots. This result is indicative of superoxide production by MDHAR6 activity with TNT.

#### 5.2.2.6 Glutathione measurements

Our previous studies on plant glutathione transferases have demonstrated that glutathione is involved in the detoxification of TNT (Gunning *et al.*, 2014). *In planta*, glutathione can be directly conjugated to TNT and may also play an indirect role in ameliorating the oxidative stress resulting from TNT toxicity. As MDHAR6 is a component of the ascorbate-glutathione recycling system in plants, glutathione levels were measured to determine whether there were perturbations in the *mdhar6* mutant.

Glutathione was measured as described in Queval and Noctor (2007). Following sample extraction and neutralization as outlined under ascorbate measurement, total glutathione was measured by recording A<sub>412</sub> increase (correlating with thionitrobenzoic acid concentration), resulting from the reaction between reduced glutathione and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). The complete assay consisted of 500 µl 0.2 M NaH<sub>2</sub>PO<sub>4</sub> 10 mM EDTA (pH 7.5), 50 µl 10 mM NADH, 50 µl 12 mM DTNB, 300 µl water, 50 µl neutralized sample or glutathione standard and 50 µl 20 U/ml glutathione reductase from Baker's Yeast (Sigma G3664) in 0.2 M NaH<sub>2</sub>PO<sub>4</sub> 10 mM EDTA (pH 7.5). The difference in A<sub>412</sub> before and after glutathione reductase addition was noted. To measure oxidized glutathione, reduced glutathione was first complexed with 2-vinylpyridine (VPD); 400 µl of neutralized supernatant was incubated at room temperature with 2 µl VPD for 30 min, and then centrifuged at 14,000 rpm for 5 min to remove the VPD-glutathione complex. For oxidized glutathione measurement, the complete assay contained 10 % (v/v) sample or standard.

While there were no differences between Col7 and *mdhar6-1* seedling leaves, *mdhar6-1* roots contained 26 % more glutathione than wild-type, although there was no difference in percentage oxidation. When whole seedlings were grown in liquid culture, and treated with TNT or a control treatment, there were no significant differences between Col7 and *mdhar6-1* ascorbate and glutathione levels and percentage oxidation. These results indicate that differences in glutathione levels do not account for the increase in TNT tolerance. To check that an altered flux via the glutathione synthesis pathway plays a role in contributing to the enhanced tolerance to TNT observed, we employed the glutathione synthesis blocker buthionine sulfoximine (BSO), which inhibits  $\gamma$ -glutamylcysteine synthetase (Griffith and Meister, 1979). Seedlings were grown for seven days on agar plates containing increasing levels of BSO in the presence or absence of TNT. In plates containing 250  $\mu$ M BSO, *mdhar6-1* seedlings remained more tolerant to 7  $\mu$ M TNT than Col-7, indicating that the tolerance of *mdhar6-1* to TNT is not coupled to a glutathione detoxification system.

### 5.2.3 Summary

MDHAR6 deficiency confers specific resistance to TNT, which is not due to differences in glutathione levels or reduced TNT uptake or a general stress response. We propose that production of superoxide by MDHAR6 with TNT as substrate is the main mechanism for TNT toxicity in plants. Reactive oxygen species accumulating in the mitochondria and plastids would easily reach damaging levels due to the cyclic nature of the reaction. These results are informative for phytoremediation applications, as well as having wider implications for possible herbicide development (Johnston *et al.*, 2015).

### 5.2.4 Introduction of *xplA/B* into *mdhar6* mutants

To investigate the potential use of plant species containing the *mdhar6* mutation for phytoremediation of RDX in TNT-contaminated soil, we engineered *mdhar6-1* Arabidopsis plants to express the RDX-degrading ability conferred by the *xplA/B* gene products. The *mdhar6-1-xplAB* plants are predicted to be able to withstand higher levels of TNT, producing higher biomass for more effective removal of RDX. We co-transformed both *mdhar6-1* and its wild-type ecotype (Col-7), with a 1:1 culture of *Agrobacterium tumefaciens* GV3101 containing the vectors *pMLBart-xplA* and *pART27-xplB*. The *pMLBart-xplA* vector confers resistance to the herbicide Basta, and *pART27-xplB* confers resistance to kanamycin. The T1 transformants were sown onto  $\frac{1}{2}$ MS (Murashige and Skoog, 1962) agar plates containing both Basta and kanamycin, to select for transformants containing both *xplA* and *xplB*. Segregating T2 lines segregating for single insertion events for each of these transgenes were selected, and homozygous, T3 lines identified for RDX and TNT-treated soil trials and liquid culture experiments.

Liquid culture screens were conducted on ten, independent homozygous *mdhar6* lines transformed with *pMLBart-xplAB* and ten, independent homozygous Col-7 lines transformed with *pMLBart-xplAB*. To set up the screens, sterilized Arabidopsis seeds were germinated on agar plates containing  $\frac{1}{2}$ MS medium and grown aseptically for 48 hours. Eight seedlings were then transferred to 100 ml conical flasks containing 20 ml liquid  $\frac{1}{2}$ MS medium amended with 20 mM sucrose. The plants were grown for three weeks on a rotary shaker (100 rpm) with 50 mmoles. $m^{-2}.s^{-1}$  white light. Plants were then dosed with 20mM sucrose containing 200  $\mu$ M TNT and 180  $\mu$ M RDX, or 180  $\mu$ M RDX only. 300  $\mu$ l of medium were removed every 24 hours, for HPLC analysis. Three replicate flasks were set up for each line. Following HPLC analysis, the rates of RDX and TNT removal were calculated, relative to untransformed, background controls.

#### 5.2.4.1 Results

The *mdhar6-xplAB* lines removed  $33\% \pm SD19$  RDX of that of unmodified Col-7 plants. The Col-7-*xplAB* lines removed  $78\% \pm SD25$  RDX, relative to unmodified, Col-7 plants. This difference in RDX uptake was unexpected and significant ( $p < 0.005$ ). There was no difference between the rate of RDX removal by the Col-7 unmodified plants and the *mdhar6-1* plants, suggesting that XplAB activity is adversely affected in the *mdhar6* background. Our hypothesis for the mode of action of MDHAR6 to confer enhanced resistance to TNT is that wild-type MDHAR6 converts TNT to a nitro-radical at sensitive locations within the cell (i.e. the mitochondria and plastids). Further studies on this could reveal information on how TNT and RDX are detoxified at the cellular/organelle level. The liquid culture studies were repeated, focussing on the lines that showed the fastest levels of RDX removal, using more replicates and testing RDX uptake in the presence and absence of TNT. Samples were analysed by HPLC.

In the flasks dosed with RDX only, the four Col-7-XplAB lines tested had removed 100, 100, 100 and 95% respectively of the RDX after two days (Figure 5.8). The four *mdhar6*-XplAB lines had removed 98, 97, 91 and 87% respectively. For comparison, at the same time point, with the wild-type Col-7, *mdhar6* mutant and no plant controls, the levels of RDX lost from the media were 24, 20 and 12%, respectively. The rate of RDX removal by the XplAB-only control line was similar to that of the Col-7-XplAB lines. These results demonstrate that the XplA and XplB enzymes are active in the Col-7-XplAB and *mdhar6*-XplAB lines. The likely reason that RDX uptake by the *mdhar6*-XplAB lines is slightly less than for the Col-7-XplAB lines is because the *mdhar6* mutation confers reduced biomass production in liquid culture conditions.

In the flasks dosed with both RDX and TNT, similar results for RDX uptake were seen, although, as observed previously, RDX uptake was delayed until all the TNT had been removed. While the *mdhar6* lines were more resistant to TNT, they did not remove TNT any faster than wild-type plants, hence this method was unable to test whether the *mdhar6* allele was present in the *mdhar6*-XplAB lines.

### 5.3 Glutathione-S-transferases (GSTs)

#### 5.3.1 Introduction

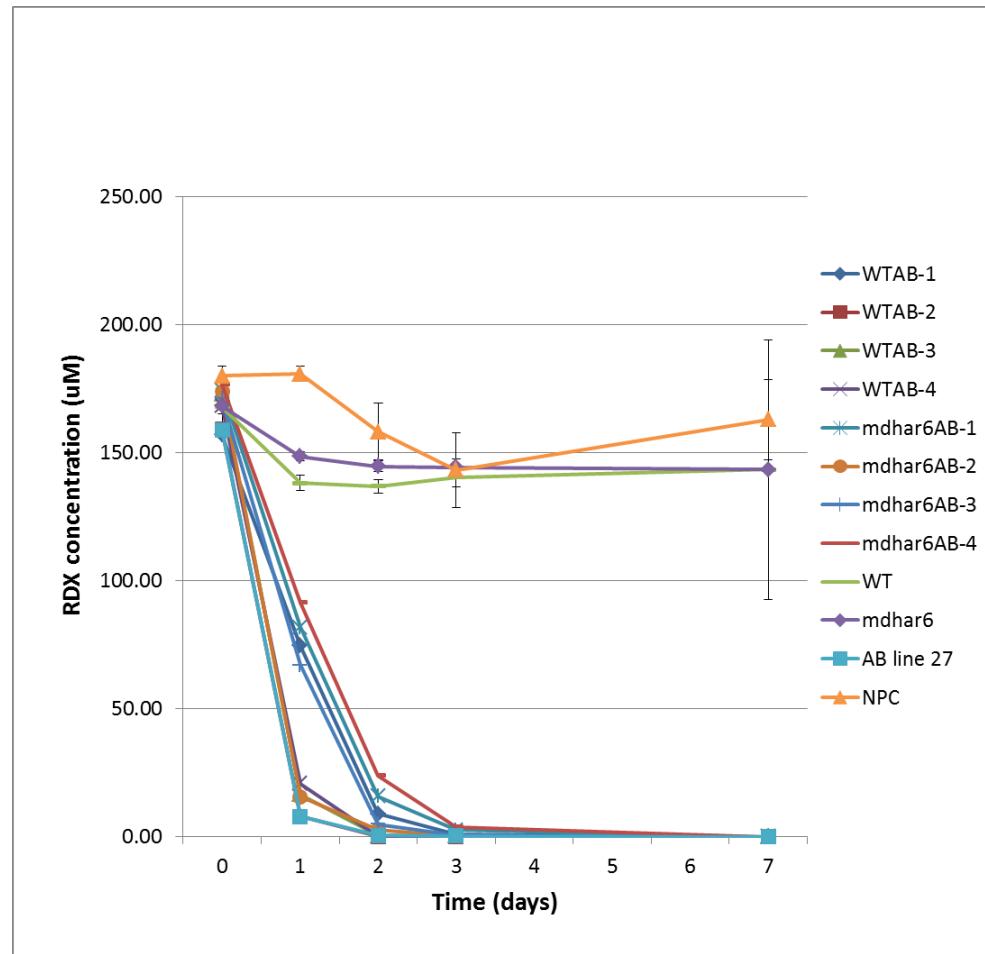
One of the main xenobiotic detoxification pathways in plants utilizes glutathione-S transferases (GSTs); enzymes that reduce the toxicity of xenobiotics by conjugating glutathione to them. Our studies using microarray analysis of the *Arabidopsis* transcriptome showed that GSTs were up-regulated in response to TNT (Gandia-Herrero *et al.*, 2008). Therefore, we investigated whether GSTs are involved in TNT detoxification in plants. The eight GSTs most up-regulated in response to TNT (all members of the Tau class) were selected for further characterization.

#### 5.3.2 Activity of Recombinant GSTs

All eight GSTs were amplified from cDNA derived from 14-day-old *Arabidopsis* seedlings exposed to TNT. The GSTs were then cloned and expressed in *E.coli*. The expressed proteins were purified and their identities confirmed by N-terminal sequencing. Details of the experimental procedures, including the GST assay conditions, may be found in the Appendix (A5.5).

Preliminary experiments using purified protein were performed, using 1-chloro-2,4-dinitrobenzene (CDNB), a model substrate for GST assays, in a spectrophotometric assay. GST-U24 and GST-U25, the two GSTs with the highest transcript expression levels in response to TNT, also yielded the highest levels of activity with CDNB as substrate (Gunning *et al.*, 2014).

Experiments to detect TNT-conjugating activity by the purified GSTs, using HPLC techniques, revealed that GST-U24 and GST-U25 also had activity towards TNT. Measurement of nitrite release, by Griess assay, showed that under the conditions tested (pH 6.5, 5 mM reduced glutathione (GSH), 200  $\mu$ M TNT and 100  $\mu$ g of protein, measured after 24 hours), GST-U24 released 7.8  $\mu$ M nitrite and GST-U25 released 40  $\mu$ M (Gunning *et al.*, 2014). These values represent 3.4 % and 20 % of the 200  $\mu$ M starting TNT concentration for GST-U24 and GST-U25, respectively. Nitrite release was not detected under these conditions for the other GSTs tested, hence subsequent studies focussed on GST-U24 and GST-U25.

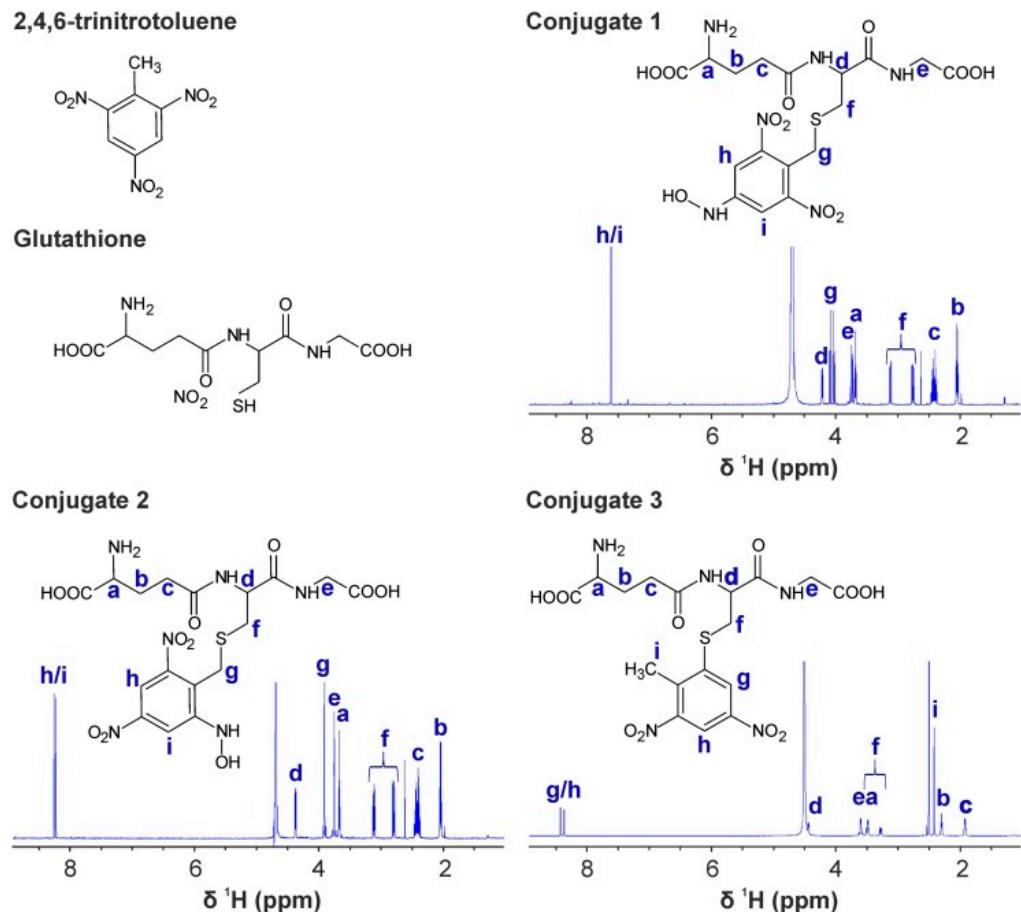


**Figure 5.8 Removal of RDX from liquid culture by wild-type Arabidopsis, *mdhar6*, *xpla/B* lines and combinations.** Medium was dosed with 180  $\mu$ M RDX only. WTAB; *xpla/B* in wild-type (Col-7) background, mdhar6AB; *xpla/B* in *mdhar6-1* background, NPC; no plant control.

Enzyme kinetics were determined, as follows. After optimization of the assay conditions, a time course experiment was performed using 100 mM potassium phosphate buffer, pH 6.5, 5 mM glutathione and 200  $\mu$ M TNT. Assays were carried out at 30°C. Under these conditions, a linear initial rate was observed for approximately 40 minutes, with transformation of almost 75% of the TNT substrate after six hours.  $V_{max}$  and related kinetic parameters were measured. A previously reported activity value for GST-U25 with CDNB was 1240 nkat.mg<sup>-1</sup> (Dixon *et*

a.l, 2009). Our assays of GST-U25 with CDNB gave an average activity of 1560 nkat.mg<sup>-1</sup>. Kinetic analysis of this reaction performed with 0 to 1000 μM of CDNB gave a K<sub>m</sub> of 30.55 μM CDNB and a V<sub>max</sub> of 28.06 μM.sec<sup>-1</sup>. GST-U25 showed no activity towards the explosives DNT, RDX and PETN (data not shown).

HPLC experiments showed that both GST-U24 and GST-U25 enzymes produced the same three GST conjugates. Mass spectrometry analysis (MS/MS) showed that conjugate 3 had an [M-H]<sup>-</sup> of 487, which corresponds to the formation of 2-glutathionyl-4,6-dinitrotoluene. In support of this, there was a concomitant release of nitrite, detected using the Griess assay, in a 1:1 ratio with the production of conjugate 3. There was no accompanying production of nitrite with the formation of conjugates 1 and 2, which both had [M-H]<sup>-</sup>'s of 518. Additional studies investigating the properties of GST-U24 and GST-U25 found that the total conjugate yield was optimal at pH 9 to 9.5, where 96-98 % of the TNT was conjugated by both GST-U24 and GST-U25. Subsequent LC/MS and NMR data indicated that conjugate 1 was C-glutathionylated 4-HADNT, conjugate 2 was C-glutathionylated 2-HADNT and conjugate 3 was 2-glutathionyl-4,6-dinitrotoluene (Figure 5.9).



**Figure 5.9** <sup>1</sup>H-NMR spectra and deduced chemical structures of TNT and the three GSH-TNT conjugates. Protons in the chemical structure, and their corresponding resonances, are labelled a to i. Assignments were completed using <sup>1</sup>H<sup>13</sup>C-HMBC, <sup>1</sup>H<sup>13</sup>C-HSQC and <sup>1</sup>H<sup>1</sup>H-COSY spectra. The key features are the resolved degeneracy of ring proton resonances (h/i and g/h) in conjugate 2 and conjugate 3 compared with conjugate 1 and the presence of a methyl singlet (i) in conjugate 3, which is absent in conjugates 1 and 2.

Purified GST-U24 produced predominantly conjugate 2, with only trace amounts of conjugates 1 and 3, while GST-U25 produced all three conjugates. For GST-U24 and

particularly GST-U25, the ratio and amounts of conjugate varied with pH and temperature, suggesting that these enzymes have catalytic flexibility.

The production of conjugate 3, (2-glutathionyl-4,6-dinitrotoluene) is of particular interest, as it demonstrates the ability of these GSTs to conjugate glutathione to TNT directly, via the removal of a nitro group (and the release of nitrite). This will de-stabilize the aromatic ring structure and may offer a possible downstream mineralization pathway, presenting the opportunity for subsequent degradation of TNT, rather than the indefinite storage of TNT-transformation products in the environment. Dinitrotoluenes are often co-contaminants at TNT-manufacturing sites, and dinitrotoluene-mineralizing bacteria have been isolated from these sites. Our findings demonstrate that manipulating GST activity *in planta* could significantly contribute towards the ability of the plant to establish in contaminated soil and mineralize TNT in the environment.

To investigate further the production of conjugate 3, we compared GST-U24 and GST-U25 with the closely-related *Arabidopsis* GST-U22, which, according to preliminary experiments, does not produce conjugate 3. Alongside this, we investigated an epsilon class GST from *Drosophila melanogaster* (DmGST6), which produces almost exclusively conjugate 3. We cloned the *Drosophila* GST into the same expression vector (*pETYSBLIC3a*) used for the *Arabidopsis* GST expression system. All four GSTs were expressed and purified.

To assay the relative levels of Conjugate 3 produced, we examined the release of nitrite by purified GST-U24 and GST-U25, alongside GST-22 and DmGST6. To do this, modified Griess assays were conducted on the purified GST proteins, using TNT as the substrate. 200 µl of sample, 200 µl of 10 mg of sulphamamide (10 mg/ml) and 40 µl of N-(1-naphthyl)ethylenediamine (10 mg/ml) were added to each well of a microtitre plate. Samples were incubated at 20 °C for 10 min and the absorbance at 540 nm measured. Nitrite was detected for GST-U24, GST-U25 and DmGST6, whereas the level of nitrite detected from GST-U22 was not significantly higher than boiled enzyme controls. In summary, across the three pH values tested (pH 6.5, 8.0 and 9.5), GST-U25 produced approximately twice the amount of nitrite released by GST-U24, while DmGST6 produced 75-100 fold more nitrite than GST-U24.

Further assays were carried out on purified DmGST6, to determine pH optima. Testing a range of pH values from 5.5 to 9.5, we found that the activity of the enzyme (µmol/min/mg protein) decreased dramatically below pH 6.0, with an optimal pH range between 8.5 to 9.0. At pH 5.5 to 7.0, DmGST6 produced exclusively conjugate 3. However, at pH range 9.0 to 9.5, conjugate 2 (1-glutathionyl-2-hydroxylamino, 4,6-dinitrotoluene) made up approximately 8 % of the total conjugates produced.

GST-U24 and GST-U25 share high identity (78 %), and we used this, in combination with published structural studies (Dixon and Edwards, 2010) to develop a site-directed mutagenesis approach to investigate the amino acid residues in GST-U25 that might be involved in catalyzing the production of conjugate 3.

GST-U24 and GST-U25 are located adjacently on *Arabidopsis* chromosome 1, possibly having evolved through gene duplication. GST-U22 is the next most closely-related (64 % and 68 % homology to GST-U24 and GST-U25, respectively), but does not have activity towards TNT. The amino-terminal domain of Tau GSTs is the site of glutathione binding (the G-site), with the carboxy-terminal domain binding the hydrophobic substrate (H-site). While sequence conservation is generally maintained at the G-site, which only binds to GSH or other very similar compounds, the H-site demonstrates much higher sequence diversity, allowing conjugation of GSH to a diverse range of hydrophobic substrates.

Using alignments with a wheat, Tau-class GST, for which the crystal structure has been solved (Thom *et al.*, 2002), along with homology modelling software packages (Swissprot, <http://web.expasy.org> and Swiss-Pdb, <http://spdbv.vital-it.ch>), we identified target amino acid

residues in the active sites of GST-U24 and GST-U25, likely to be important for substrate specificity. Site directed mutagenesis was then carried out on GST-U24 and GST-U25, with the aim of identifying the amino acid residues responsible for the production of conjugate 3. Within the GST-U24 sequence, we have identified certain amino acids that differ from GST-U25. In the H site, Ala-12 was identified and in the G-site, Asn-107, Ala-115, Ile-209 and Arg-212 were different. These residues were replaced with their counterparts from in GST-U25. That is, Pro-12 was substituted in the H site and in the G-site, Asn-107, Ala-115, Val-209 and Leu-212 were substituted. Reciprocal substitutions were also made in GST-U25.

A number of variants were produced, expressed in *E.coli* and purified, using the protocols described in Gunning *et al.* (2014). These variants were screened with TNT as the substrate, using Griess assays to detect nitrite production and HPLC to profile conjugate specificities.

The results showed that for wild-type GST-U24, the major product was conjugate 2. However, a mutant form of GST-U24 containing all five substitutions produced approximately equal amounts of all three conjugates, along with a 1.6-fold overall increase in conjugating activity.

### 5.3.3 Over-expression of GSTs in *Arabidopsis*

*GSTs U1, U3, U4, U7, U22, U24 and U25* were cloned into the *pART7/27* binary vector system (Gleave, 1992), under the control of the CaMV35S promoter and *ocs* terminator, for near-constitutive expression in *Arabidopsis*. The constructs were introduced into *Arabidopsis* by *Agrobacterium*-mediated transformation, using the floral dipping method (Clough & Bent, 1998). Transformants were selected on kanamycin, and seed of the T4 generation was used for analysis.

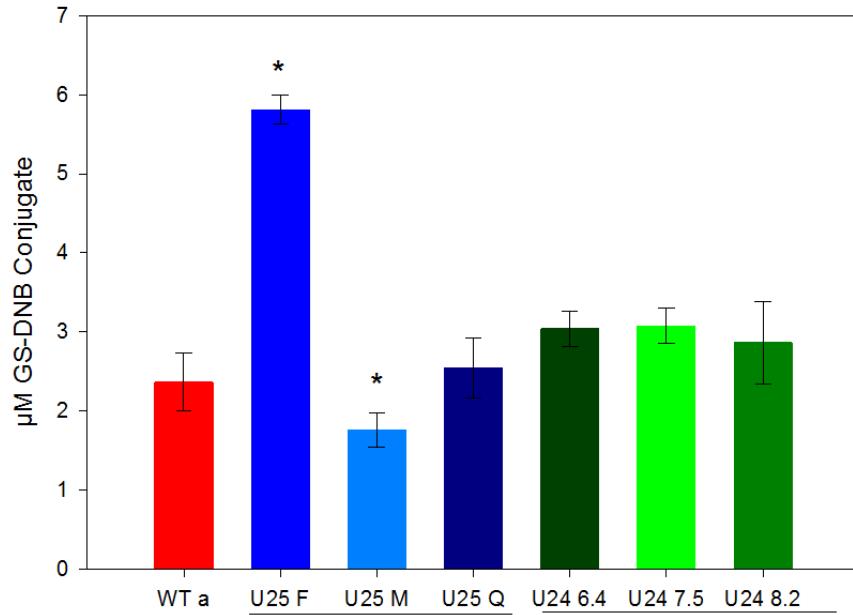
Gene expression studies were carried out, in the presence of TNT. Real-time RT-PCR experiments showed that *GST-U24* and *GST-U25* had the highest increases in expression, in response to TNT.

Further RT-PCR was carried out on the *35SGST-U24* and *35SGST-U25* lines, using RNA extracted from leaf tissue. Transcript levels of the respective *GSTs* were assessed for each line, alongside those of the alternate *GST*. That is, *35SGST-U24* lines were tested for levels of *GST-U24* and *GST-U25*, and *vice versa*. Although the primers used for cloning and those used for RT-PCR were shown to differentiate clearly between the two closely-related genes, it is possible that some co-regulation may occur. For this reason, both sets of lines were also measured for expression of *GST-U22*. *GST-U22* is also induced by TNT and of those genes up-regulated by TNT treatment, it is the most similar to *GST-U24* and *GST-U25*, with 64 % and 68 % identity to them, respectively.

The three *35SGST-U24* lines all showed high levels of over-expression. However, most of these lines did not have increased levels of *GST-U25*. The *35SGST-U25* lines all over-expressed *GST-U25*, although line M was a rather weak over-expressor compared with lines F and Q. *GST-U24* expression was not altered in the *35SGST-U25* background.

It is unlikely that these transcript levels directly correspond to the amount of protein produced, so to quantify the levels of protein expression, Western blot studies were carried out. Antibodies were acquired from Dr David Dixon (University of Durham, UK) and Prof. Peter Goldsborough (Purdue University, USA). These antibodies were raised against the maize protein ZmGSTU1-2 and the *Arabidopsis* protein AtGST-U19, respectively, and were expected to work with the Tau GSTs used in this study. Despite optimization of the experimental conditions, very little signal was produced by the positive control of pure protein at high concentrations, and no signal was observed for the *35SGST-U* lines. Extracts of GST-overexpressing plants have previously been shown to have increased activity (relative to wild-type) in spectrophotometric CDNB assays (Takesawa *et al.*, 2002), thus we performed

CDNB assays for GST activity (Figure 5.10). Interestingly, *35SGST-U25* line M, which had the lowest GST expression, had less activity than wild-type. Other lines exhibited a general trend of increased activity towards CDNB, although only *35SGST-U25* line F (the line that also had the highest transcript level) had significantly more activity than wild-type.



**Figure 5.10 CDNB assay of protein extracts from *35SGSTU* lines.**

Assays contained 10 µg protein, 1 mM GSH and 1mM CDNB in 100 mM potassium phosphate pH 6.5. Change in absorbance at 340 nm was measured over 1 min. Significant difference from wild-type (WT) is shown by an asterisk and determined by Dunnett's test ( $p<0.05$ ). Results are the means of three technical replicates. Error bars represent SD.

To investigate whether increased levels of GST-U24 and GST-U25 activities confer increased abilities to detoxify TNT and thus withstand its phytotoxic effects, we conducted a range of hydroponic and soil-based experiments using *Arabidopsis* lines over-expressing GST-U24 or GST-U25. Seven homozygous independent *35SGST-U24* and *35SGST-U25* lines were used.

Plants were assayed for GST activity, using CDNB as substrate. Rosette leaves from three-week-old plants were homogenized in protein extraction buffer (0.1 M Tris-HCl, pH 8, 2 mM EDTA, 1 mM DTT, 50 g/kg PVPP). After centrifugation, GST activity was measured spectrophotometrically at 340 nm. The increase in absorbance due to reaction product accumulation was measured over 1 min and data were normalized against no-enzyme controls. Following confirmation that GST-U24 and GST-U25 were actively over-expressed, seedlings were grown on solid agar medium containing a range of rates of TNT. Root lengths were measured, as an indicator of the relative tolerance of plants towards TNT. Seedlings were photographed at 9, 14 and 20 days and root lengths and root areas measured using Image J and Image Pro software. Analysis of the data revealed that both the GST-U24 and GST-U25 over-expressor lines produced significantly more root biomass than wild-type plants, when grown on TNT-containing medium.

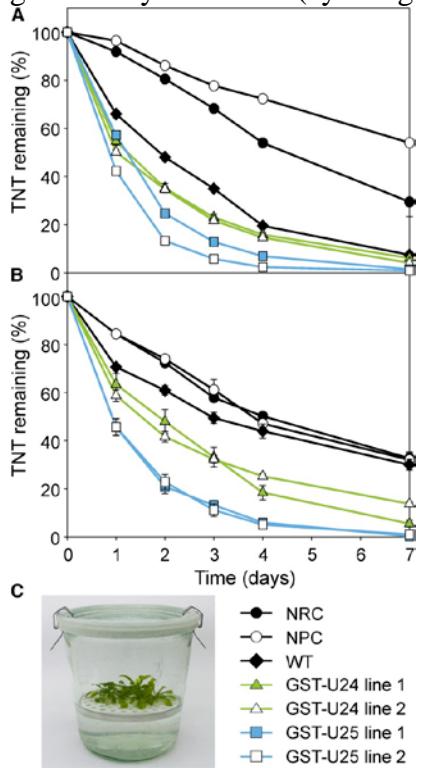
### **5.3.3.1 Hydroponic Studies with the 35SGST-U lines**

Arabidopsis plants were grown in liquid culture, enabling us to follow the uptake of TNT from the medium by wild-type and *GST-U24* and *GST-U25* transformed lines. Imbibed seeds were germinated on agar plates containing half-strength ( $\frac{1}{2}$  MS) medium. Seven-day-old seedlings were transferred to 100 ml flasks (eight plants per flask), containing 20 ml of  $\frac{1}{2}$  MS plus 20 mM sucrose. Five replicate flasks were set up for each of three independent homozygous lines of the *GST-U24*- and *GST-U25*-over-expressors, with unmodified wild-type plants as a control (seven plant lines in total). After 14 days, the medium was replaced by 20 mM sucrose containing 200  $\mu$ M TNT. Samples were taken periodically over five days and analyzed using HPLC. The results showed that only at the 24 hour time point did the *GST-U24* and *GST-U25* over-expressing lines remove significantly more TNT from the medium than the unmodified lines. At the end of the experiment, there were no discernible differences in the appearance of the plant lines.

When Arabidopsis plants are grown in liquid culture, their physiology is altered. The waxy cuticle layer is reduced and much of the plant is submerged in the culture medium. These factors are likely to enable TNT to penetrate all the plant organs equally. These conditions are far-removed from the in-field conditions of a plant growing on a military training range, where only the roots are in contact with TNT in the soil. We also know that, in soil-grown plants, TNT is localised mainly in the root tissue and, importantly, both *GST-U24* and *GST-U25* are expressed predominantly in the roots (in the root endodermis and root epidermal atrichoblasts, respectively). Therefore, to enable TNT uptake and metabolite analysis under more realistic conditions, plants were grown hydroponically on plastic rafts. The rafts were made from circular, lightweight plastic discs, 70 mm in diameter and 6 mm thick, with approximately 100 holes (3-4 mm diameter) drilled into each disc. Sterile, stratified Arabidopsis seeds (8 per raft) were pipetted onto the holes filled with  $\frac{1}{2}$  MS agar, and placed in sterile glass jars containing liquid  $\frac{1}{2}$  MS medium. The seeds were allowed to germinate and the plants were grown aseptically and hydroponically for 20 days, prior to dosing with 30 ml of  $\frac{1}{2}$  MS medium containing 50 or 100  $\mu$ M TNT. Samples were taken every 24 hours over seven days and analyzed by HPLC. Five replicate jars were set up for each of two independent homozygous lines of the *GST-U24*- and *GST-U25*-overexpressors, with unmodified wild-type plants as control (five plant lines in total). The results showed that the *GST-U24* plant lines removed the TNT 50 % faster than unmodified plants, whereas the *GST-U25* lines removed the TNT more than twice as rapidly as the unmodified lines (Figure 5.11). Growing Arabidopsis plants in liquid culture also enabled us to extract and analyze the TNT conjugates formed in the plants. For the TNT-conjugate analysis, the plants were grown in the same conditions and metabolites were extracted from the whole plants at 0, 4, 6, 8 & 24 hour time points. Analysis was carried out using HPLC and LC/MS. The results showed the presence of free TNT in the plant tissues 4 hours after dosing.

Within 24 hours, most of the TNT in the tissues was conjugated. The major TNT-conjugation product had a molecular weight of 375, corresponding to o-glucosylated hydroxyl-amino dinitrotoluene (HADNT), identified previously during UDP-glycosyltransferase conjugation analyses. At much lower levels, the LC/MS analyses also confirmed the formation of glutathionylated products in the transgenic plants. Conjugate 2 was detected solely in the *GST-U24* over-expressing plants, and found to increase with time, whereas conjugate 3 was detected in the *GST-U25* over-expressing plants and also increased over time. The formation of conjugate 3 in the *GST-U25* line is likely to reflect the physiological conditions in the plant cells, as we have shown that the ratio and amounts of conjugates varies dramatically in *GST-U25* according to pH.

LC/MS did not reveal the presence of metabolites resulting from the processing of glutathionylated TNT (cysteinglycine,  $\gamma$ -glutamylcysteine or cysteine derivatives).



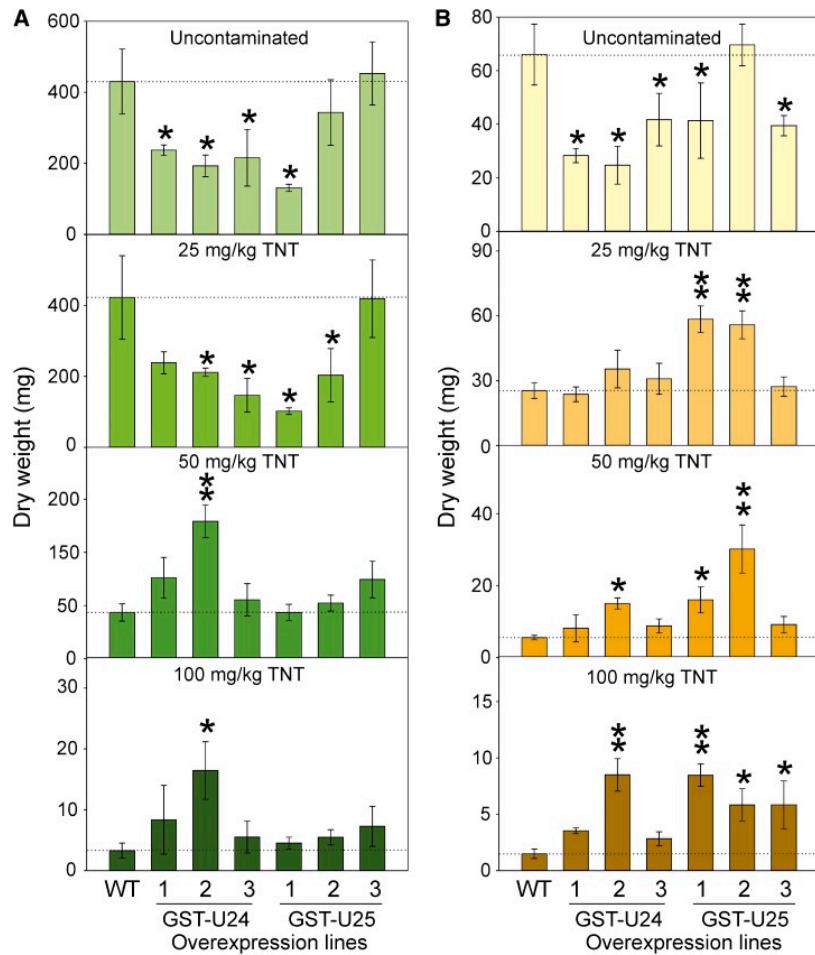
**Figure 5.11 Rate of TNT uptake from hydroponic culture by wild-type (WT), *GST-U24-* and *GST-U25*-overexpressing *Arabidopsis* plants.** A and B, Three-week-old plants were grown on rafts with roots submerged in  $\frac{1}{2}$ MS medium, then dosed with 50  $\mu$ M (A) or 100  $\mu$ M (B) TNT. C, Experimental set-up. NRC, No-raft control; NPC, no-plant control. Results are means of five biological replicates  $\pm$  SE. (from Gunning *et al.*, 2014)

### 5.3.3.2 Soil-based experiments with the 35SGST-U lines

On military training ranges, contamination levels of between 100 and 1000 mg kg<sup>-1</sup> TNT have been reported (Talmage *et al.*, 1999; Jenkins *et al.*, 2006) To test the detoxification abilities of the *GST-U24* and *GST-U25* over-expression lines, we chose TNT concentrations of 25, 50 and 100 mg/kg; levels which unmodified *Arabidopsis* plants could tolerate, while still being representative of the levels found on training ranges.

The soil experiments were set up as follows. A 30 mg/ml stock solution of TNT in acetone was aliquoted onto 50 g dry sand in 2 litre polypropylene tubs. For uncontaminated soil, a volume of acetone equivalent to that used for soil treated with the highest level of TNT was applied. The acetone was evaporated overnight and the tubs placed on a rotating mixer, with a 35 mm glass marble added to each tub to aid mixing, for 1 hr. Following mixing of the sand and TNT, 450 g of soil (Levington's F2 compost) were added and the tubs mixed overnight. Equal amounts of soil were weighed into 5 cm high plastic pots and five 5-day-old seedlings were planted into the soil. The plants were placed in a growth cabinet with 180  $\mu$ mol.m<sup>-2</sup>.s<sup>-1</sup> light and 12 hr photoperiod, at 18 °C (dark) and 21 °C (light).

When the plants were six weeks old, the root and aerial tissues were harvested and dry biomass weights determined (Figure 5.12). The results showed that, when grown in untreated soil, both shoots and roots were significantly reduced ( $p < 0.05$ ) in all three of the *GST-U24* lines, and to a lesser degree, in the *GST-U25* over-expressing lines, compared to the unmodified lines.



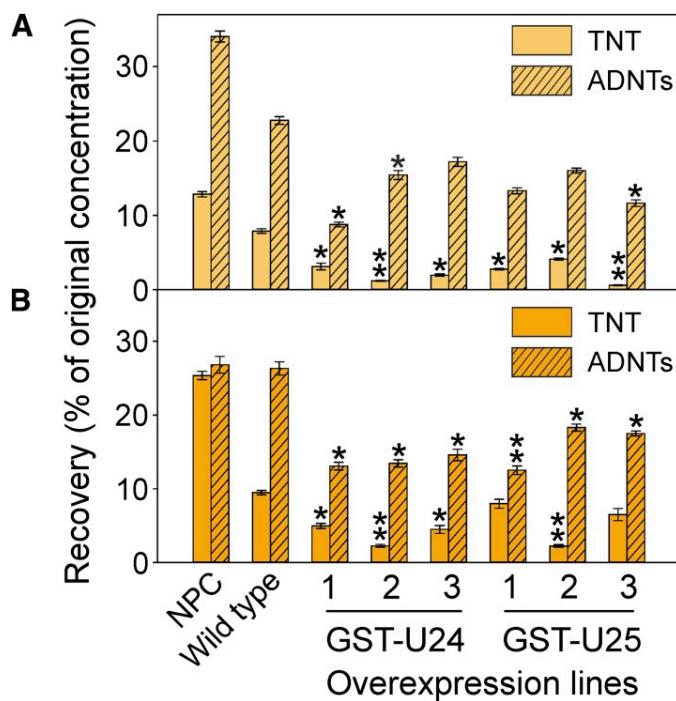
**Figure 5.12 Shoot (A) and root (B) biomasses of *Arabidopsis* plants grown in TNT-treated soil.** Wild-type (WT) and GST-U24- and GST-U25-over-expressing *Arabidopsis* plants were grown in soil contaminated with a range of TNT concentrations for 6 weeks. Results are means of eight biological replicates  $\pm$  SE. Asterisks denote statistically significant differences from the wild-type: \* $p < 0.05$ , \*\* $p < 0.01$ .

When the plants were grown in increasing concentrations of TNT-treated soil, the tissue dry weights of the *GST-U24* and *GST-U25* over-expressing lines increased such that they were similar to, or greater than, unmodified plants grown in soil at the same TNT concentration. The data were normalized to account for the unexpectedly reduced, relative to wild type, biomasses of the *GST-U24* and *GST-U25* over-expression lines grown in untreated soil. These normalized data revealed more clearly that, when grown in soil treated with 50 and 100 mg/kg TNT, both the shoot and root biomasses of the *GST-U24* and *GST-U25* over-expressing lines were significantly higher than unmodified plants. At the highest concentration of TNT tested, the combined mean shoot and root dry biomasses of the *GST-U24* over-expressing lines were, respectively, 6.4 and 7.9-fold higher than unmodified plants. For the *GST-U25* over-expressing lines, the mean shoot and root biomasses were 2.9 and 6.4-fold higher, respectively.

The levels of TNT and amino dinitrotoluenes in the soil were measured after these experiments. Along with TNT, significant levels of the transformed products 2- and 4-amino dinitrotoluenes (ADNTs) were found. It is likely that these were from TNT transformed by soil microorganisms over the course of the experiment.

The 100 mg/kg TNT concentration was phytotoxic to the extent that root growth was so poor that the bulk of the soil in these pots was not colonized by the plant roots. It is likely that relatively little TNT would have been removed by such small root biomass, so levels of TNT and ADNT were not measured in these pots. With the 25 mg/kg treatment, the levels of TNT in the soil were 60-85% less for the *GST-U24* lines and 48-92% less for the *GST-U25* lines, relative to the soil in pots containing untransformed plants. With the 50 mg/kg treatment, the levels of TNT in the soil were 48-76% less for the *GST-U24* lines and 16-77% less for the *GST-U25* lines (Figure 5.13).

The levels of ADNTs in both the 25 and 50 mg/kg TNT-treated soil experiments were also lower than in the soil from pots containing untransformed plants. However, the ratio of TNT:ADNT was less for the *GST*-over-expressing lines, indicating that these lines removed TNT in preference to ADNT. This is in agreement with our studies using purified *GST-U24* and *GST-U25*, which show that these enzymes have activity towards TNT, but not towards ADNTs.



**Figure 5.13 Levels of nitrotoluenes recovered from TNT-treated soil.** Wild-type and *GST-U24*- and *GST-U25*-overexpressing *Arabidopsis* plants were grown alongside no-plant control (NPC) pots of soil treated with 25 mg kg<sup>-1</sup> (A) or 50 mg kg<sup>-1</sup> (B) TNT for 6 weeks, and then TNT and ADNTs were extracted from the soil. Results are means of eight biological replicates ± SE. Asterisks denote statistically significant differences from the wild-type: \**p* < 0.05, \*\**p* < 0.01. (Gunning *et al.*, 2014)

As mentioned above, when the over-expressing lines were grown in untreated soil, the root and shoot biomasses were significantly reduced compared with untransformed plants. A possible reason for this 'yield drag' effect could be that the over-expression lines use glutathione to conjugate other compounds in the absence of TNT, with the result that the depletion of the glutathione pool negatively affects metabolism. To investigate this, we measured the levels of glutathione in six-week-old rosette leaves from plants grown in untreated soil, and in soil treated with 50 mg/kg TNT. The results showed that the levels of total glutathione were not significantly different between untransformed and over-expressing

lines in uncontaminated or TNT-treated soil, although total glutathione levels were all lower in the leaves of plants grown in TNT-treated soil. While the leaves from untransformed plants grown in TNT-treated soil had 29 % less total glutathione, the over-expression lines exhibited larger reductions in total glutathione levels (35, 46, 47, 53, 56 and 65 % for the lines tested). These data suggest that when the over-expression lines are grown in TNT-treated soil, they are depleting more of the available glutathione and are using this for direct conjugation to TNT. There was no change in the ratio of reduced:oxidized glutathione in any of the results. As most of the TNT was initially localized in the root, we also wanted to measure glutathione content in roots. This was not possible for soil-grown plants, but measurements were made on roots grown vertically on agar plates without TNT. There was no significant difference between the levels of glutathione in the roots of the over-expressing lines and those of the untransformed plants. Thus, we concluded that the yield drag exhibited in the over-expression lines is not the result of altered glutathione pools.

Members of the Tau class of plant GSTs, which include GST-U24 and GST-U25, have been shown to exhibit glutathione-dependent peroxide (GPOX) activity. The GPOX activity catalyzes the reduction of lipid hydroperoxides to the respective monohydroxyalcohols, and is involved in conferring stress tolerance. We assayed purified GST-U24 and GST-U25 and found that both these enzymes have GPOX activity. It is possible that the yield drag is due to some unknown, deleterious effect resulting from increased GPOX activity (Gunning *et al.*, 2014).

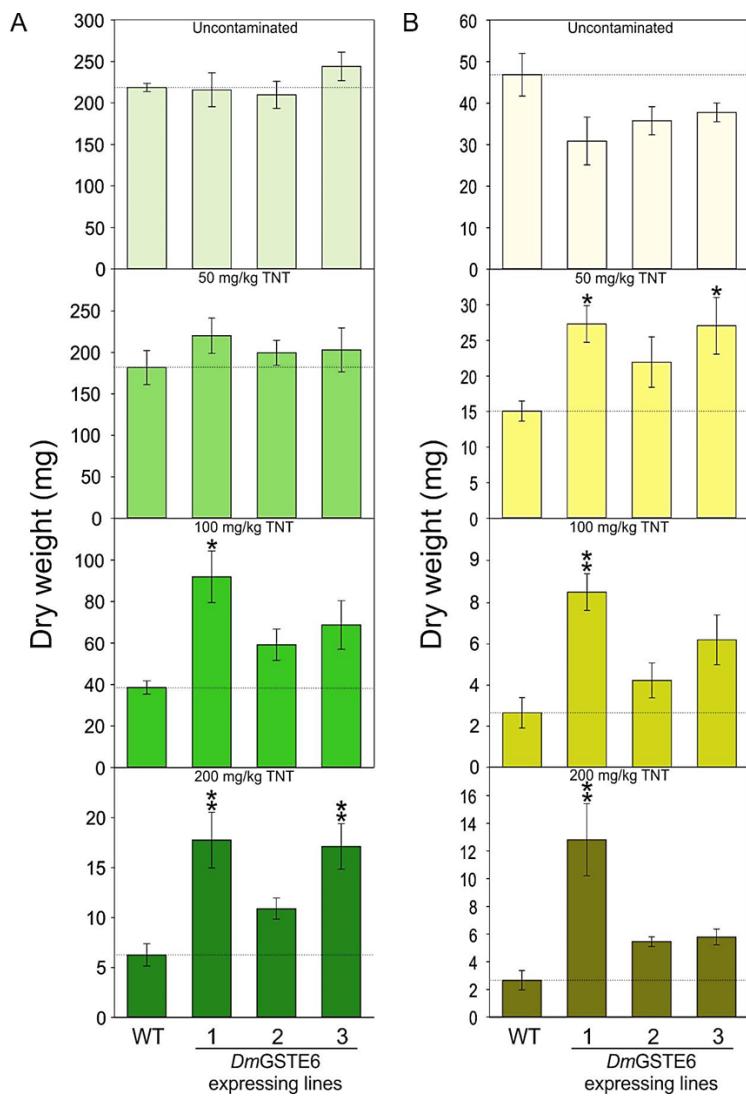
### 5.3.3.3 Expression of Drosophila GST (DmGST6) in Arabidopsis

As described previously, assays on purified DmGST6, using TNT as substrate, revealed that DmGST6 had significantly more activity towards TNT than either endogenous *Arabidopsis* GST-U24 or GST-U25. Notably, DmGST6 produces almost exclusively conjugate 3 (2-glutathionyl-4,6-dinitrotoluene), which we consider a favourable activity, as this compound may represent a future opportunity for TNT mineralization. Since *Arabidopsis* plants over-expressing GST-U25 produce more root and shoot biomass than unmodified plants when grown in TNT-contaminated soil, it would be expected that expression of DmGST6 in *Arabidopsis* could confer considerably more resistance to TNT than plants expressing GST-U25. To investigate this, we transformed wild-type *Arabidopsis* with *DmGST6*, under the control of the near constitutive CaMV-35S promoter. Experiments were carried out with homozygous lines in the T3 generation. Assays on crude root and leaf extracts, using CDNB as substrate, showed that the three lines with the highest activity had 1.4 to 2.4-fold increases in activity in the roots and 2.1 to 2.8-fold increases in activity in the shoots.

Soil-based experiments were conducted with the *DmGST6*-expressing lines, in soil treated with a range of rates of TNT (0, 50, 100 and 200 mg/kg). After six weeks' growth, the plants were harvested and the root and shoot biomasses were found to be significantly higher in the *DmGST6*-expressing lines (Figure 5.14). For example, on soil treated with 100 mg/kg TNT, the root biomasses of the three transgenic lines were 2.8, 1.6 and 1.9-fold higher than the wild-type. Shoot biomasses were 2.4, 1.5 and 1.8-fold higher, respectively.

The levels of extractable TNT in soil from the pots treated with 50 mg/kg were measured after six weeks' plant growth. The levels of TNT in soil from all three *DmGST6* lines were significantly lower than the levels in the soil where the wild-type plants had been growing. During the six-week experiment, much of the TNT in the soil was converted to ADNTs by the activity of soil microorganisms. The levels of extractable ADNT were also found to be significantly lower in the *DmGST6* lines than in the wild-type lines (wild-type =  $48.6 \pm 3.5\%$ , *DmGST6* lines 1, 2 and 3 =  $30.5 \pm 5.9\%$ ,  $35.2 \pm 4.1\%$  and  $30.0 \pm 9.9\%$ , respectively).

We also transformed the *mdhar6-1* mutant (section 5.2.1) with *DmGST6*, to attempt to enhance further the TNT resistance phenotype.



**Figure 5.14 Shoot (A) and Root (B) biomasses of one-week-old *Arabidopsis* seedlings grown for six weeks in soil treated with a range of TNT concentrations.** WT, untransformed; dGST/1-3, independent homozygous lines expressing *DmGSTE6*. Results are means of eight biological replicates  $\pm$  SE. Asterisks denote statistically significant from the WT: \* $P<0.05$ , \*\* $P<0.01$ .

### 5.3.3.4 Production of DmGSTE6 activity in a $\gamma$ -glutamyl cysteine synthetase ( $\gamma$ -ECS) background

When the levels of glutathione (GSH) were measured in the *GST-U24* and *GST-U25* over-expressing lines growing in TNT-treated soil, we found that these lines had GSH levels significantly lower than those of untransformed plants. GSH levels were unaffected in the absence of TNT. Given the preliminary data suggesting that DmGSTE6 had greater activity towards TNT than GST-U24 and GST-U25, it is possible that GSH could be limiting in *DmGSTE6*-expressing *Arabidopsis* lines grown on TNT-treated soil.

In *Arabidopsis*, evidence demonstrates that  $\gamma$ -glutamyl cysteine synthetase is the limiting step in the synthesis of GSH. Dr Om Parkash, (University of Massachusetts, Amherst, USA),

kindly provided  $\gamma$ -ECS over-expressing lines that have increased levels of GSH and are more resistant to arsenic as a result. To investigate whether increasing the pool of available GSH can enhance TNT uptake and detoxification, we have transformed these  $\gamma$ -ECS over-expressing lines with a *DmGSTE6* construct. The *DmGSTE6* gene was re-cloned into *pMLBart*, to enable selection on Basta.

#### 5.3.3.5 Downstream processing of TNT-GST conjugates

Our studies indicated that the conjugation of TNT to glutathione is a major step in the detoxification of TNT in plants, but the subsequent fate of these TNT conjugation products is unknown. As discussed in our recent paper in Plant Signalling & Behaviour, (Rylott *et al.*, 2015a), the fate of TNT conjugation products can be proposed by the extrapolation of studies on herbicides such as monobromobimane (mBB), which is conjugated to glutathione. The mBB-glutathione conjugates are sequestered into the vacuole and in *Arabidopsis* root vacuoles, the N-terminal degradation products, Cys-Gly-mBB and  $\gamma$ -Glu-mBB, are produced by  $\gamma$ -glutamyl transpeptidase 4 (GGT4). Studies by Naoko Ohkama-Ohtsu (2007) and others on *ggt4* mutants have demonstrated that GGT4 is responsible for the majority of mBB-glutathione derived conjugates and that C-terminal degradation of glutathione is not significant. The *ggt4* mutants were also found to be less resistant to the herbicides mBB, 2,4-dichlorophenoxyacetic acid and metachlor. Given the parallels between mBB-glutathione and TNT-glutathione conjugates, it is possible that *ggt4* lines are also compromised in their resistance to TNT. Naoko Ohkama-Ohtsu kindly donated the *ggt4* line, as well as *ggt1* and the double mutant *ggt1ggt4*. (GGT1 is expressed in the extra-cellular space). These lines are being bulked up for seed prior to preliminary studies to measure root lengths of wild-type and *ggt* lines on medium containing TNT.

#### 5.3.3.6 Testing the effect of exogenous glutathione on *in planta* TNT detoxification.

We have reported previously (Gunning *et al.*, 2014) that glutathione could be a factor limiting TNT detoxification by plant glutathione transferases. When *Arabidopsis* plants were grown for six weeks in soil treated with 50 mg/kg TNT, the leaves from wild-type plants had 29 % less total glutathione than those grown in untreated soil. Moreover, plants over-expressing GST-U24 and GST-U25 had even less total glutathione than wild-type plants when grown in TNT-treated soil (*GST-U24* lines, 35, 46 and 47 %, respectively; *GST-U25* lines, 53, 56 and 65 % respectively). Our subsequent studies using *Arabidopsis* plants expressing *DmGSTE6* further supported the theory that glutathione might be a limiting factor. Enzyme kinetics data for purified *DmGSTE6* demonstrated that this had a higher affinity and  $V_{max}$  towards TNT as a substrate, than reported for *GST-U24* and *GST-U25* (Gunning *et al.*, 2014). However, there were no increases in biomass and root length of *DmGSTE6*-expressing *Arabidopsis* lines grown in TNT-treated soil, relative to *GST-U24* and *GST-U25*-over-expressing plants.

To investigate the role of glutathione in TNT detoxification *in planta*, wild-type and *DmGSTE6* plant lines were grown in liquid culture, and exogenously supplied with glutathione. To do this, sterile *Arabidopsis* seeds were germinated on  $\frac{1}{2}$ MS plus 20 mM sucrose agar plates, and eight, one-day-old seedlings were transferred to each 100 ml conical flask containing 20 ml liquid  $\frac{1}{2}$ MS plus 20 mM sucrose. The plants were grown under 20  $\mu\text{mol. m}^{-2}. \text{s}^{-1}$  light on a rotary shaker at 130 rpm until 14 days old. After this time, the medium was replaced with 20 ml of 20 mM sucrose containing 250  $\mu\text{M}$  TNT and 0, 100, 250 or 1000  $\mu\text{M}$  glutathione. The removal of TNT from the liquid culture medium was measured over five days using HPLC.

In the 0, 100 and 250  $\mu\text{M}$  glutathione treatments, the *DmGSTE6* lines removed TNT faster than wild-type plants. However, interestingly, the addition of 100 or 250  $\mu\text{M}$  glutathione increased the rate of TNT uptake compared to plants dosed with TNT alone. (After 24 hours,

in the TNT-only flasks, there was  $51.0 \pm 2.8\%$  TNT remaining in wild type flasks, and  $32.8 \pm 6.3\%$  in the *DmGSTE6* flasks. In the flasks dosed with TNT plus 100  $\mu\text{M}$  glutathione,  $36.5 \pm 4.6$  and  $17.3 \pm 2.4\%$  of the TNT remained in the wild-type and *DmGSTE6* lines, respectively).

Both wild-type and *DmGSTE6* lines in the flasks dosed with the highest concentration of glutathione (1000  $\mu\text{M}$ ), showed signs of toxicity, removing less than 50 % of the TNT after five days, and appearing necrotic.

The health of the plants at the end of the experiment was quantified by measuring chlorophyll levels in seven-day-old plants. Plants subjected to 100 and 250  $\mu\text{M}$  glutathione treatments were found to have more chlorophyll than plants dosed with TNT alone. (Wild-type and *DmGSTE6* plants dosed with TNT only had  $136.8 \pm 4.00$  and  $288.3 \pm 11.8 \mu\text{g}$  respectively of chlorophyll per g fresh weight. Wild-type and *DmGSTE6* plants dosed with TNT plus 100  $\mu\text{M}$  glutathione had  $120.0 \pm 16.00$  and  $189.4 \pm 20.3 \mu\text{g}$  chlorophyll per g fresh weight). These exciting results demonstrate a link between glutathione and TNT detoxification *in planta*.

Measurement of glutathione in whole plant tissues from wild-type plants 24 hours after treatment showed that, in the presence of TNT, the wild-type plants contained  $96.7 \pm 8.4 \text{ nmole.g}^{-1}$  fresh weight total glutathione. As expected, the presence of exogenously supplied 100  $\mu\text{M}$  glutathione, in the absence of TNT, increased the glutathione levels in the plants. However, the application of glutathione together with TNT returned the level of endogenous glutathione to a level similar to that in the TNT-only treatment. Both oxidized and reduced levels of glutathione were measured and no differences in their ratios were found across the different treatments.

When glutathione levels were measured in the *DmGSTE6*-over-expressing line, increased amounts of total glutathione, relative to wild-type, were found in all treatments (250  $\mu\text{M}$  TNT only =  $186.6 \pm 12.9$ ; 100  $\mu\text{M}$  glutathione only =  $185.4 \pm 23.7$ ; 250  $\mu\text{M}$  TNT and 100  $\mu\text{M}$  glutathione =  $148.3 \pm 19.0$ ). The percentages of reduced glutathione were significantly higher than wild-type in all treatments.

The results above indicate that detoxifying TNT draws on glutathione reserves. In plants, discreet glutathione pools exist within subcellular organelles as well as the cytosol. The endogenous GSTs U24 and U25 and *DmGSTE6* are all expressed only in the cytosol and there may be more complex regulation of glutathione pools in place. Furthermore, expression of *DmGSTE6* may not be subject to the same regulation as endogenous GSTs in Arabidopsis.

### 5.3.3.7 Summary

The current hypothesis for TNT-detoxification in plants is that there is a rate-limiting nitroreductase step, followed by glucosyltransferase activities. Our studies demonstrated the contribution of GSTs to the TNT detoxification pathway in Arabidopsis. We showed that over-expression of plant GSTs confers enhanced resistance to TNT, alongside an increased ability to remove this pollutant from the environment and detoxify it. GST-U25 can directly conjugate glutathione to TNT, via the removal of a nitro group, producing 2-glutathionyl-4,6-dinitrotoluene. The removal of a nitro group from TNT is highly desirable because it destabilizes the aromatic ring, allowing TNT to be degraded and mineralized, instead of stored in the environment. Our research shows that selecting or manipulating plants with increased GST activity, especially for the production of conjugate 3, could significantly contribute towards the ability of plants to grow in contaminated soil and clean up TNT from polluted military sites.

## Task 6 Transformation of Grasses

### 6.1 Introduction

The experiments described in section 5.1 demonstrated that plants can be engineered to withstand the toxicity of TNT and remediate RDX contamination, through the transgenic expression of the bacterial gene for detoxification of TNT (*nfsI*) and the bacterial genes for RDX degradation (*XplA* and *XplB*). The aforementioned experiments were performed in the model plant species *Arabidopsis*, which would be unsuitable for large-scale phytoremediation applications.

Therefore, the primary goal of the research in task 6 was to transfer the technology to plant species that are suitable for use on live-fire military training ranges. The most practical plants for this purpose are grasses, which are easily seeded over large areas and are resistant to fire and heavy equipment.

We chose Western wheatgrass (*Pascopyrum smithii*) and switchgrass (*Panicum virgatum L*) as representatives of grass species frequently planted on training ranges in the United States. In addition, we transformed a model grass species, creeping bentgrass (*Agrostis stolonifera*), as a useful subject for greenhouse studies of RDX degradation.

The *xplA*, *xplB* and *nfsI* genes were cloned into plasmid vectors and used to transform wheatgrass, creeping bentgrass and switchgrass. Considerable time and effort were invested in the optimization of the transformation and regeneration protocols for each species. Details of the experimental procedures and the results are summarized in the following sections.

## 6.2 Tissue culture of grasses

### 6.2.1 Creeping bentgrass

Tissue culture of creeping bentgrass resulted in calli comprising a mixture of embryogenic and soft callus. These mixed calli could easily be induced to form healthy plantlets on suitable regeneration medium.

### 6.2.2 Switchgrass

Using established protocols, the shells of mature caryopses of switchgrass were etched with sulphuric acid, surface sterilized with 20% bleach and cultured on callus induction medium (MS salts with 22.5 µM 2,4-6-BAP and 30 g/L maltose at pH5.5). After two weeks' culture, watery callus appeared at an induction efficiency of about 80%. After culture for 1.5 months, small white globular calli, characteristic of embryogenic callus, developed from the soft callus at a rate of about 1-2%. The embryogenic calli were sub-cultured on callus induction medium in preparation for propagation and transformation.

### 6.2.3 Western wheatgrass

Three types of callus were produced when western wheatgrass was grown in tissue culture; embryogenic (friable) callus, ‘soft and mixed’ callus and watery callus. Watery callus produced no regeneration. Soft and mixed callus regenerated with high efficiency, but we were not able to transform this type of callus using particle bombardment (biolistics). This could be because the surface of the soft and mixed callus is covered by non-embryogenic cells, preventing penetration of the ballistic particles to the embryogenic cells. Friable callus was transformable using the ballistic method, but the regeneration efficiency was very low. To date, *Agrobacterium* transformation has not been successful with western wheatgrass.

To generate embryogenic callus suitable for transformation, seeds of western wheatgrass were surface sterilized in 20% bleach for 20 min and then cultured on solid CICG medium (MS basal medium supplied with 2.5 mg/l 2,4-D + 0.1 mg/l 6-BAP) in darkness, for two months. Embryogenic calli were separated and sub-cultured on fresh CICG medium, with transfers at 3-week intervals.

### **6.3 Preliminary transformation experiments**

Detailed protocols for *Agrobacterium* culture, embryogenic callus infection and selection and regeneration of transgenic plants have been described previously (Lee *et al.*, 2011; Li and Qu, 2011).

#### **6.3.1 Creeping bentgrass**

Embryogenic callus was transformed using *Agrobacterium* strain EHA105, harbouring the binary vector construct *pRCS2-bar-ABNR* that contains the *xplA*, *xplB* and *nfsI* genes, with *bar* as the selection marker gene, conferring resistance to ppt (DL-phosphinothricin, otherwise known as glufosinate). Six ppt-resistant calli were obtained. PCR using genomic DNA isolated from the calli as templates confirmed the integration of *xplA*, *xplB* and *nfsI* into the creeping bentgrass nuclear genome. Several plantlets were regenerated, but these all had an albino phenotype.

Embryogenic callus of creeping bentgrass was also transformed with *Agrobacterium* (EHA105) harbouring the binary vector construct pCambia1301 (as selection vector) and *pRCS2-hpt-ABNR* containing the *xplA*, *xplB* and *nfsI* genes, with *hpt* as the selection marker gene, conferring resistance to hygromycin. Four hygromycin resistant calli were obtained. Two of these were albino, but two were healthy. PCR using genomic DNA isolated from the calli as templates confirmed the integration of *xplA*, *xplB* and *nfsI* into the creeping bentgrass nuclear genome. RT-PCR analysis confirmed expression of the *hpt*, *xplA*, *xplB* and *nfsI* genes, although the expression level of *nfsI* was very low.

#### **6.3.2 Western wheatgrass**

Newly-induced embryogenic calli of western wheatgrass were bombarded with *pRCS2-bar-ABNR* and ppt resistant calli regenerated. Five green plantlets developed. Embryogenic calli were also bombarded with *pRCS2-hpt-ABNR*. 22 hygromycin-resistant calli were obtained and these were cultured in liquid regeneration medium for the induction of plantlets.

### **6.4 Reconstruction of transformation vectors**

It is likely that the *ocs* promoter driving the expression of the selection marker gene in our constructs was not sufficiently strong in grass species, preventing healthy growth on the selection media. Therefore, it was decided to reconstruct the transformation vector using the well-tested 35S viral promoter. The 35S expression cassette (including the 35S promoter, 35S terminator and multiple cloning site) was released from *pSAT4a* by digestion with *AgeI* and *NotI* and used to replace the *ocs* expression cassette in *pSAT1a*, to produce *pSAT1a-35S*. The hygromycin resistance gene *hpt* was released as a *EcoRI-BamHI* fragment and inserted into *pSAT1a-35S*, to produce *pSAT1a-35S-hpt*. Then the *hpt* expression cassette was cut from *pSAT1a-35S-hpt* by *AscI* and inserted into the binary vector *pRCS2-ABNR*, making *pRCS2-35S-hpt-ABNR*. This vector was used to transform switchgrass, western wheatgrass and creeping bentgrass, with some, limited, success.

For example, embryogenic switchgrass calli were transformed with *pRCS2-35S-hpt-ABNR* and many plantlets were obtained after the hygromycin resistant calli were transferred to regeneration medium (MS with 0.2 mg/L NAA, 1 mg/L BAP, 0.5 mg/L GA and 30 g/L maltose), without hygromycin. These plantlets were transferred to regeneration medium with hygromycin at 100 mg/L, for selection. 47 plantlets grew quickly on the selection medium. However, PCR performed on DNA isolated from these lines revealed that only 2 of these were actually transformed with *xplA-xplB-nfsI*.

The sequence of vector *pRCS2-35S-hpt-ABNR* was checked and the *xplA* gene was found to be inserted into the vector in the reverse direction. This would result in the production of no XplA protein. The incorrect insertion of the *xplA* gene into the vector was likely due to the presence of an undocumented restriction site in the *pSAT* vector system (specifically *pSAT6a*) that we used to construct the 4-gene stacked binary vectors used for transformation of grasses. The *xplA* gene was cloned by PCR and inserted into *pSAT6a* as a *Hind3-Hind3* fragment, to produce *pSAT6a-xplA*. Then the expression cassette, including the promoter of the rubisco small subunit (*rbc*), the coding sequence of *xplA* and the *rbc* terminator, was released from the *pSAT6a-xplA* plasmid and inserted into the *pRCS2-35S-hpt-ABNR* plasmid, replacing the old XplA expression cassette, to produce the new binary vector, *pRCS2-ANBR-HR*. This vector was used to transform Arabidopsis, as a positive control. The *xplA-xplB-nfsI* -transformed Arabidopsis exhibited RDX degradation ability.

Calli of creeping bentgrass and switchgrass were subsequently transformed with *pRCS2-ABNR-HR*. For creeping bentgrass, many plantlets were regenerated from calli on RECBG medium (MS with BAP at 1 mg/L) with hygromycin at 100 mg/L and were cultured further on selection medium. The insertion of the *xplA*, *xplB*, *nfsI* and *hpt* genes was tested by PCR. Gene expression was tested by RT-PCR and western blots with antibodies against XplA and XplB. RDX was taken up from liquid medium by the transformed plants and degraded at higher rates than wild-type. Wild-type and transformed seedlings were cultured in liquid MS medium containing 50 µM TNT, and cultured, with shaking, for 20 days. The transformants continued growing, while the wild-type plants withered.

Switchgrass transformed with *pRCS2-35S-hpt-ABNR-HR* produced 21 healthy plants that could grow on the selection medium. PCR showed that nine of these contained the *xpla*, *XplB*, *nfsI* and *hpt* genes.

## 6.5 Use of monocot promoters

### 6.5.1 Vector construction

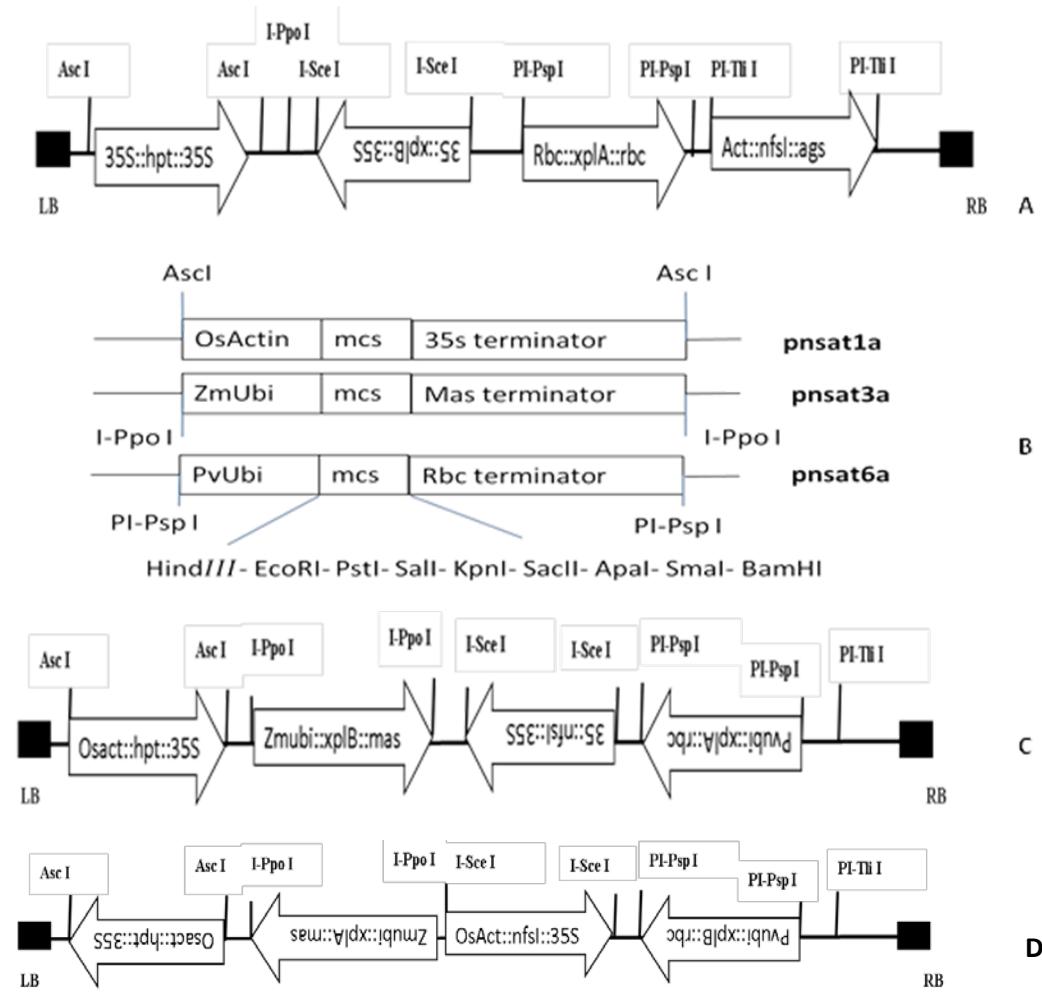
To improve the results by enhancing the expression levels of *xplA*, *xplB* and *nfsI* in grasses, promoters specific to monocot plants were cloned and constructed in *pSAT* vectors. The promoter of the actin gene of rice (*Oryza sativa (Posact)*), the promoter of the ubiquitin gene of maize (*Zea mays (Pzmubi)*) and the ubiquitin gene of switchgrass (*Ppvubi*) were cloned by PCR, using DNA from the vector *pANIC 5A* (Mann *et al.*, 2012) as the template. The *Posact* cassette was constructed into *pSAT1a* to replace the *ocs* promoter used previously, producing the new vector *pNSAT1a*. The *Pzmubi* cassette was constructed into *pSAT3a* to replace the *mas* promoter, producing the new vector *pNSAT3a*. The *Ppvubi* cassette was constructed into *pSAT6a*, to replace the *rbc* promoter, producing the new vector, *pNSAT6a*.

The *GFP* and *GUS* reporter genes were constructed into *pNSAT1a*, *pNSAT3a* and *pNSAT6a* separately, to make constructs that would serve as expression and technical controls.

Expression cassettes containing the *hpt* selection marker gene from *pNSAT1a-hpt*, the *GFP* gene from *pNSAT3a-GFP* and the *GUS* gene from *pNSAT6a-GUS* were constructed into the binary vector *pRCS2*, to produce the control vector, *pRCS2-3G-6G*.

The *xplA*, *xplB* and *nfsI* genes were constructed into *pNSAT6a*, *pNSAT3a* and *pNSAT4a*, respectively, and then the expression cassettes of *hpt* from *pNSAT1a-hpt*, *XplA* from *pNSAT6a-XplA*, *XplB* from *pNSAT3a-XplB* and *nfsI* from *pSAT4a-NR* were constructed into binary vector *pRCS2*, to produce the stacked vector *pRCS2-NABNR*. This vector was transferred into *Agrobacterium* strain EHA105 by the freeze-thaw method and the resulting strain grown in LB medium with 50 mg/L rifampicin, 100 mg/L spectinomycin and 300 mg/L streptomycin. This vector was then used to transform switchgrass and creeping bentgrass.

Another vector, named *pEDLZ2014*, was developed from *pNABNR* by switching the position of *XplA* and *XplB*, and replacing the 35S promoter for *nfsI* expression with the actin promoter from rice (Figure 6.1).



**Figure 6.1 Construction of *pRCS2-ABNR-HR*, *pRCS2-NABNR*, and *pEDLZ2014* vectors for transformation of grasses.**

**A:** T-DNA region of the binary vector plasmid pRCS2-ABNR-HR. The RDX degradation gene *xplA*, flavodoxin reductase gene *xplB*, and TNT detoxifying nitroreductase gene *nfsI* were constructed into the *pSAT* cloning vector system. The expression cassettes, including *xplA*, *xplB*, and *nfsI*, were released from the *pSAT* cloning vectors using homing restriction enzymes *PI-PspI*, *I-SceI*, and *PI-TliI* separately and then inserted into the binary vector *pPZP-rcs2* to produce pRCS2-ABNR-HR.

**B:** T-DNA region of the binary vector plasmid pRCS2-NABNR. New *pSAT* vectors were developed to enhance multiple gene expression in monocot grasses. The *Osactin*, *Zmubi*, and *Pvubi* monocot promoters were used to replace the promoters in the *pSAT* vector to produce *pNSAT1a*, *pNSAT3a*, and *pNSAT6a*.

**C:** *Hpt*, *xplA*, *xplB*, and *nfsI* genes were constructed into *pNSAT1a*, *pNSAT6a*, *pNSAT3a*, and *pNSAT4a*. The expression cassettes of these genes were integrated into binary vector *pPZP-RCS2* to produce pNABNR.

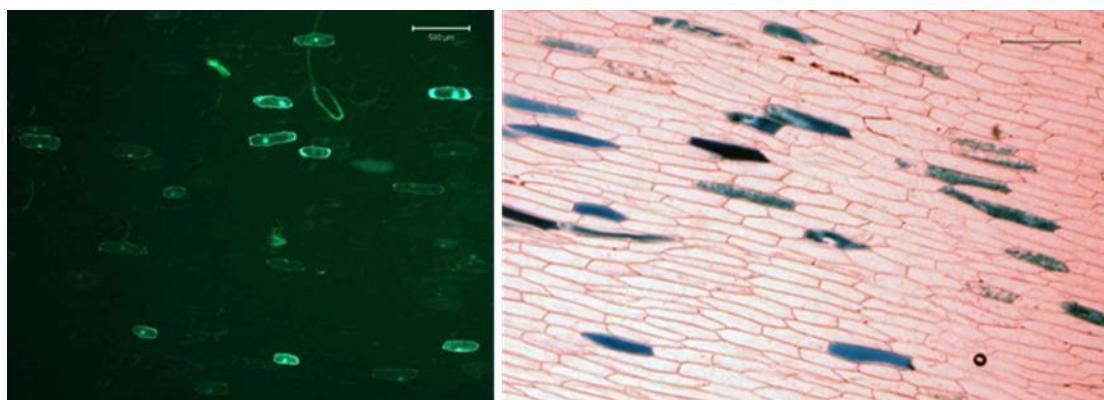
**D:** The vector *pNABNR* was modified to produce *pEDLZ2014*.

Abbreviations: 35S, CaMV 35S; rbc, Rubisco small subunit; act, actin; ags, agropine synthase; *OsActin*, promoter of actin gene of *Oryza sativa*; *ZmUbi*, promoter of ubiquitin of *Zea mays*; *PvUbi*, promoter of ubiquitin gene of *Panicum virgatum* (switchgrass); RB left border; RB right border.

### 6.5.2 Functional evaluation of the new vectors by expression of reporter genes in epidermal cells of onion

As described above, the reporter genes *GFP* and *GUS* were separately inserted, as *EcoRI-BamHI* fragments, into *pNSAT1a*, *pNSAT3a* and *pNSAT6a*, to produce *pNSAT1/3/6a-GFP/GUS* vectors that were used to bombard epidermal cells of onion. Biolistic bombardment was carried out using a PDS1000He biolistic gun (Bio-Rad), with 10 cm target distance and rupture pressure of 1100 psi. The bombarded onion was wrapped with wet paper towels and kept in a culture vessel at room temperature in darkness for two days before observation.

*GFP* expression was observed using a fluorescence microscope (Figure 6.2). The *GFP* signal was found to accumulate in the cytosol, especially near the nucleus. Histochemical staining was used to observe *GUS* expression. The *GUS* protein also accumulated in the cytosol. The transient expression of reporter genes in onion epidermal cells demonstrated that the promoters used in our constructs were able to drive the expression of foreign genes in monocot plants.



**Figure 6.2 Functional evaluation of the *pNSAT* vectors by transient expression of reporter genes in epidermal cells of onion.**

A. Fluorescence microscopy showing GFP expression in the cytosol of epidermal cells of onion following particle bombardment with *pNSAT1a-GFP* (*Osactin::GFP::35s*).

B. Histochemical staining for GUS expression in the cytosol of epidermal cells of onion following particle bombardment with *pNSAT3a/6a-GFP* (*Zmubi::GUS::Mas; Pvubi::GUS::rbc*).

## 6.6 Transformation experiments using constructs with monocot promoters

### 6.6.1 Materials and methods

#### 6.6.1.1 Molecular analysis of transgenic plants

For polymerase chain reaction (PCR) analysis, a DNeasy plant mini kit (Qiagen) was used to purify DNA from hygromycin resistant plants. PCR reactions were carried out by amplifying the expression cassette regions of the *hpt* gene, including parts of the promoter and terminator sequences. Primer sequences are shown in table A6.1 in the Appendix.

For analysis of gene expression by reverse transcription PCR (RT-PCR), total RNA was extracted from the leaves of transformed plants, using the RNeasy plant mini kit (Qiagen). cDNA was transcribed using M-MLV reverse transcriptase (Promega).

### **6.6.1.2 Protein extraction and immunoblot analyses**

Total protein from leaves was isolated with QB buffer (100mM KPO<sub>4</sub> buffer, pH 7.8), 1mM EDTA (pH 8.0), 1% Triton X-100, 10% glycerol, 1mM DTT) supplied with 2 mM phenylmethylsulfonyl fluoride (PMSF), separated by Mini protean TGX Tm gels (Bio-Rad), and then transferred to PVDF membrane (Millipore corp., Bedford, MA). The proteins were detected using specific antibodies previously generated in rabbit. Detection was performed with the anti-rabbit IgG-alkaline phosphatase (Sigma-Aldrich).

### **6.6.1.3 RDX uptake by transformed switchgrass and creeping bentgrass.**

Wild-type and transformed grass plants with similar biomass and at the same development status were selected and cultured in 5 ml ½ MS medium (without sucrose), under 16 hr light, 8 hr dark photoperiod at 25°C for 15 days. RDX was added to the medium (20 mg RDX/l) and the RDX concentration in the medium assayed daily. After 15 days' culture, the RDX concentration in the plant tissue was also analyzed. 100 mg plant tissue were collected and freeze-dried, using a Fast Prep 24 (MP Biomedicals, LLC., Solon, USA). Powdered samples were immersed in 1 ml methanol:water (1:1) and incubated for 12 hours at room temperature, without shaking. The tubes were then centrifuged twice at 13000 rpm for 10 min. The supernatant (800 µl) was collected for HPLC analysis.

### **6.6.1.4 HPLC quantification of RDX.**

RDX concentrations were analyzed using a modular Waters HPLC system, consisting of a Waters 717 auto-sampler, two Waters 515 HPLC pumps and a Waters 9926 photodiode array detector. A 4.6-by 250-mm Waters C18 column was used for separation, and concentrations were determined based on absorbance at 240 nm. External standards were run with each sample batch. Peak integrations were conducted using Millennium 32 software (Waters, Milford, MA).

### **6.6.1.5 Propagation of transformed switchgrass**

Transformed switchgrass was transferred to soil and cultured in the greenhouse. Stem nodes from fully-developed plants were collected and surface-sterilized in 20% bleach for 20 min. The sterile nodes were cultured on solid RMOT medium (MS basal medium plus 1 mg/l 6-BAP and 0.1 mg/l NAA) under light for 2 months, to induce cluster shoots.

### **6.6.1.6 Transformation of callus of western wheatgrass using the biolistic method**

Embryogenic callus was exposed to high osmotic treatment for 4 hours before bombardment. The calli were transferred to an area 5 cm in diameter at the centre of a Petri dish containing callus-inducing medium supplemented with 0.6 M mannitol. DNA-coated gold particles (0.6 µm diameter) were prepared with *pEDLZ2014*. Bombardment was carried out using a Bio-Rad PDS-1100/He biolistic delivery system at 1100 psi, using a 9 cm target distance. After 24 hours, the calli were transferred to CICG medium without mannitol. After one week, they were transferred to selection medium (CICG medium with 80 mg/l hygromycin). After three weeks' growth, resistant calli were transferred to fresh selection medium for a further 3 weeks. The hygromycin resistant calli were propagated on CICG medium dosed with hygromycin at 50 mg/l and selected calli were transferred to REG medium (MS basal medium with 0.2 mg/l NAA + 1 mg/l BAP + 0.5 mg/l gibberellic acid), with hygromycin at 50 mg/l for regeneration. DNA and RNA samples were isolated from regenerated plantlets for molecular analysis.

## **6.6.2 Results**

### **6.6.2.1 Production of transgenic grasses and molecular analyses**

Embryogenic calli of creeping bentgrass were infected with *Agrobacterium* strain EHA105 harbouring *pRCS2-NABNR* and placed on CICG medium containing the selection agent hygromycin. Hygromycin resistant calli were obtained after three weeks. The resistant calli were transferred to REG medium with hygromycin, to develop plantlets. Green and healthy plantlets were subjected to PCR screening using NSAT3A primers. Clear bands of the expected size (approximately 1.6 kb) confirmed integration of the transgenes. RT-PCR revealed detectable transcript for *hpt*, *xplA*, *xplB* and *nfsI*, for all the transgenic plant lines tested. Western blot analysis using antibodies to XplA and XplB confirmed the presence of these proteins.

For switchgrass, the very friable type II embryogenic callus (Burris *et al.*, 2009) was used for infection with *Agrobacterium* EHA105 harbouring *pRCS2-NABNR*. Switchgrass callus was apparently naturally resistant to the selection agent when cultured on callus induction medium with hygromycin at 100 mg/l for two weeks as first round selection. Many calli survived and proliferated, but were killed during the second and third rounds with hygromycin at 200 mg/l. Calli growing vigorously on the selection medium during the third round of screening were transferred to regeneration medium with hygromycin at 50 mg/l for plant development. After two months, healthy plants were transferred to soil. PCR, RT-PCR and western blot analyses were carried out to confirm the integration and expression of the genes.

Embryogenic callus of western wheatgrass was bombarded with *pEDLZ2014* plasmid DNA and screened on callus induction medium with hygromycin at 75 mg/l for two months. The hygromycin resistant calli were transferred to regeneration medium containing 50 mg/l hygromycin, to produce plantlets. After one month, small plantlets were transferred to MS medium with hygromycin at 50 mg/l for further development and rooting. DNA was isolated (separately) from the resistant callus and plantlets and PCR was performed to confirm the transformation.

In previous experiments with western wheatgrass, we found that the regeneration efficiency was low. Many of the calli developed into unhealthy plantlets, which subsequently died. With the new technique, and vectors constructed with monocot promoters, 71 hygromycin resistant calli were obtained, and nine of these tested positive with PCR. Fully developed plantlets were obtained from the nine separate lines and these are being cultured on MS medium for further analysis.

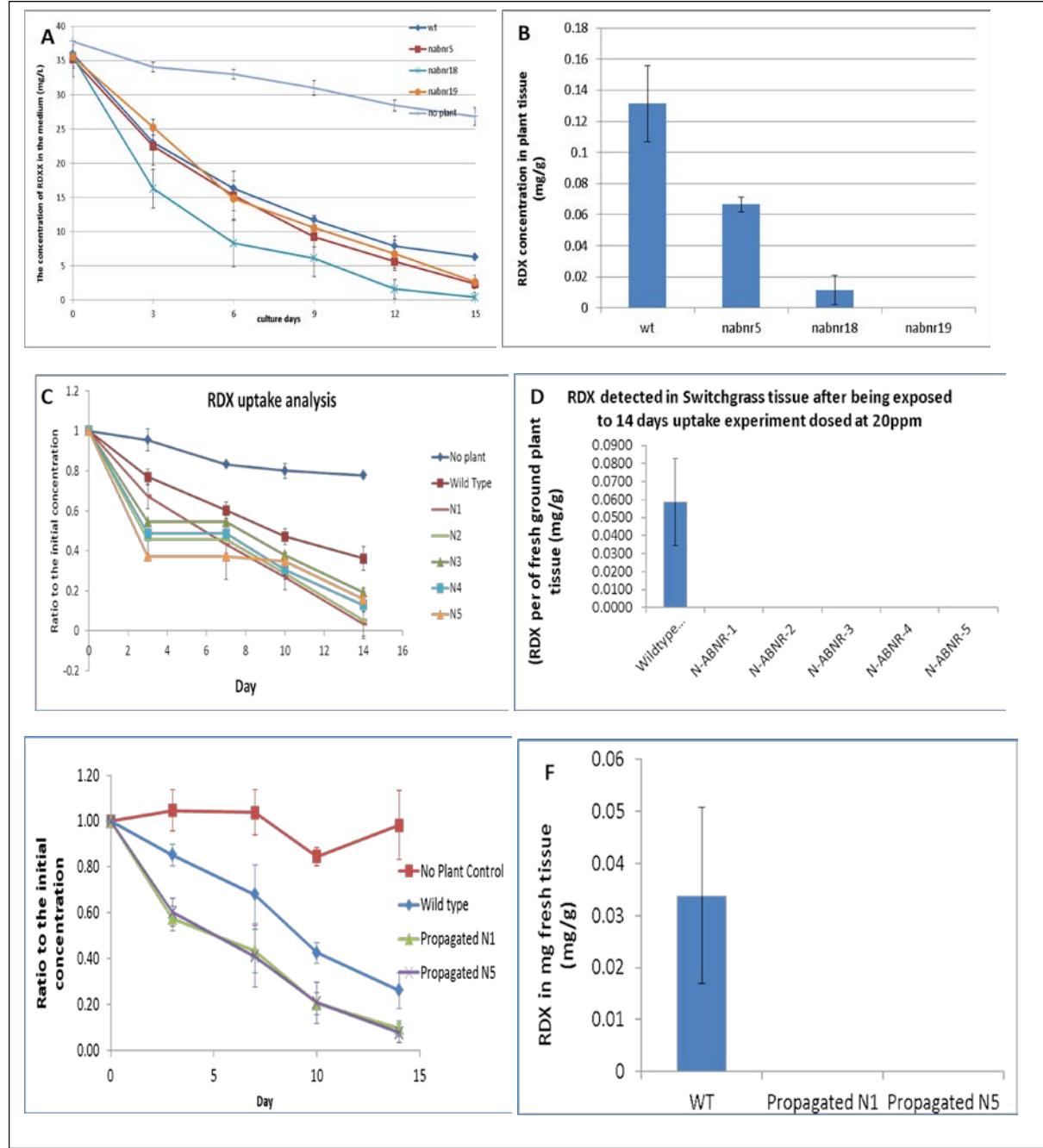
## 6.7 RDX uptake and degradation by transformed grasses

To simulate the uptake of RDX by plants on a training range, transgenic and wild-type switchgrass and creeping bentgrass were cultured in liquid media in open tubes for high efficiency of transpiration. RDX concentrations in all media decreased over a period of two weeks, though uptake was faster with the transgenic plants expressing *xplA* and *xplB* than with the wild-type plants (Figure 6.3).

Following uptake by wild-type plants, RDX is translocated to the leaf tissues (Brentner *et al.*, 2010). In wild-type plants, RDX in leaf tissue is partially reduced to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) and hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX). Further transformation of RDX, MNX and DNX results in the formation of formaldehyde, methanol and carbon dioxide, through light-mediated mechanisms (Van Aken *et al.*, 2004). However, these activities are minimal and wild-type plants have not been shown to degrade RDX to any environmentally significant extent. Thus, RDX accumulates in the leaf tissue, inhibiting further uptake and limiting the capacity of wild-type plants to remove RDX from the soil (Just & Schnoor, 2004; Winfield, 2004). Furthermore, senescence and die-back of plants in the winter season would likely return accumulated RDX to the soil environment.

In our experiments, RDX was extracted from leaf tissue and analyzed by HPLC after each uptake experiment. RDX accumulated in wild-type creeping bentgrass (Figure 6.3) and

switchgrass, while much lower levels of RDX were detected in the tissues of most of the transformed plants expressing *XplA*/*XplB*. These results show that transformed lines of creeping bentgrass and switchgrass were able to take up and degrade RDX, hence demonstrating that expression of *XplA* and *XplB* in grass can confer the ability to phytoremediate RDX.



**Figure 6.3 Uptake and degradation of RDX by *xplA/B*-transformed grasses.** (A) Concentration of RDX in culture medium with creeping bentgrass. (B) Concentration of RDX remaining in creeping bentgrass tissue after 15 days' uptake. (C) Concentration of RDX in culture medium with switchgrass. (D) Concentration of RDX remaining in tissue of switchgrass after 14 days' uptake. (E) RDX concentration in liquid medium with propagated switchgrass, transgenic lines N1 and N5. (F) Concentration of residual RDX in plant tissue of switchgrass after 14 days' uptake and degradation. All the data are means  $\pm$ SE of three replicates except for the data in E and F, which had 6 replicates. RDX levels in N1 and N5

tissues was below detection limits. Creeping bentgrass was grown in liquid  $\frac{1}{2}$  MS medium containing RDX at 40 mg/L and switchgrass plants were grown in liquid  $\frac{1}{2}$  MS medium dosed with RDX at 20 mg/L. The concentration of RDX in the medium was determined by HPLC analysis of media samples.

The *NABNR*-transformed switchgrass lines were propagated by inducing cluster shoots from stem nodes, and these propagated plants were also used to repeat the RDX uptake test. The results were similar to those of the previous experiments, proving that the RDX uptake and degradation properties of the transformed switchgrass lines are suitably robust. This is the first time that grasses have been transformed for degradation of an organic pollutant.

Subsequently, further hydroponic experiments were carried out with creeping bentgrass, to support the above results. 5 g (fresh weight) of grass stems with blades and one to three internodes were bunched together and placed in 250 ml glass conical flasks containing 200 ml water or 200 ml 150  $\mu$ M RDX. Ten replicate flasks were set up for each line (eight transgenic lines, plus wild-type and no plant control flasks). The flasks were placed on rotary shakers at 100 rpm, under light at 100  $\mu\text{mol.m}^{-2}.\text{sec}^{-2}$ . Samples of the growth medium were taken at regular time intervals and RDX uptake measured using HPLC.

The results showed that, over 28 days, the levels of RDX in the flasks containing the transgenic creeping bentgrass declined steadily from 150  $\mu$ M to between 110 and 20  $\mu$ M RDX. The RDX concentration in the flask containing the wild-type bentgrass reached 65  $\mu$ M after 28 days. The rates of RDX uptake by the transgenic lines were lower than those predicted from similar experiments with the same lines, hence the levels of expression of XplA were analysed using RT-PCR and western blots, and found to be very low. It is possible that the ability to degrade RDX has been lost, due to transgene silencing that may have occurred during propagation. We are now re-transforming creeping bentgrass to test new lines and generate seed as a more reliable germplasm store.

## Task 7

### Analysis of tissue-specific promoters

#### 7.1 Introduction

The use of tissue-specific promoters is predicted to have certain advantages over the constitutive 35S promoter when designing transgenic plants. Tissue-specific promoters may increase expression in the targeted area beyond that of the 35S promoter and may reduce the metabolic toll of producing the target protein throughout the plant and/or confine detoxification metabolites. In addition, tissue-specific promoters can reduce gene silencing problems, which can be triggered by the use of multiple copies of identical promoters.

Preliminary studies at the University of York showed that fluorescent TNT analogues are taken up by the root hairs and stored in the root epidermal cell walls, presumably so that these toxic metabolites are confined promptly and the overall exposure of the plant to toxicity is reduced. We have used the *Agrobacterium* root-specific promoter *RolD* to determine whether high levels of NR and XplA activity targeted solely to the roots can enhance removal of TNT and RDX from the root zone of transgenic Arabidopsis plants. We also tested the Arabidopsis ubiquitin promoter (*UBQ*), which provides strong, constitutive transgene expression and assessed its performance in comparison with the 35S promoter.

#### 7.2 Materials and Methods

##### 7.2.1 Plasmid construction and *Arabidopsis* transformation

The binary construct, *pBIN-AXR1*, containing the root-specific expression promoter *RolD* from *Agrobacterium rhizogenes* was obtained from Prof. Ottoline Leyser's group, formerly at the University of York. The *AXR1* gene was replaced with the nitroreductase gene *nfsI*, to

drive root-specific expression of *nfsI*. To produce *pUBQ-nfsI*, the 35S promoter region of *pART7* was replaced with *UBQ* using the *NotI* and *XhoI* sites and *nfsI* was cloned into the *EcoRI* and *BamHI* sites, downstream of the *UBQ* promoter.

Both constructs were introduced into wild-type, *xplA* and *xplA/B* Arabidopsis lines, using floral dipping. *pRoLD-nfsI* also confers kanamycin resistance as the plant selectable marker. The *xplB*-containing lines are also kanamycin resistant. TNT could be used as a screen to identify TNT-resistant plants transformed with the *pRoLD-nfsI* construct.

### 7.2.2 Liquid culture experiments

Arabidopsis seeds were surface-sterilized and germinated on agar plates containing half-strength MS medium, then transferred to 100 ml conical flasks containing 20 ml ½MS and 20 mM sucrose (eight seedlings per flask). When the plants were four weeks old, the medium was replaced with water containing 250 µM TNT. Aliquots of media were removed at set time points and TNT levels were determined using HPLC.

### 7.2.3 Soil studies

Arabidopsis seeds were surface-sterilized, then germinated, on agar plates containing ½MS, with 20 mM sucrose and 7 µM TNT (as described in Gandia-Herrero *et al*, 2008). Root lengths of 7-day-old seedlings were measured. TNT-treated soil studies were conducted as described in Rylott *et al.*, (2011b). Briefly, five-day-old seedlings were transferred to plastic pots containing 30 g TNT-treated soil and grown for six weeks in a growth chamber with a 12 hour photoperiod. Levels of TNT in plant extracts were determined using EPA Method 8330.

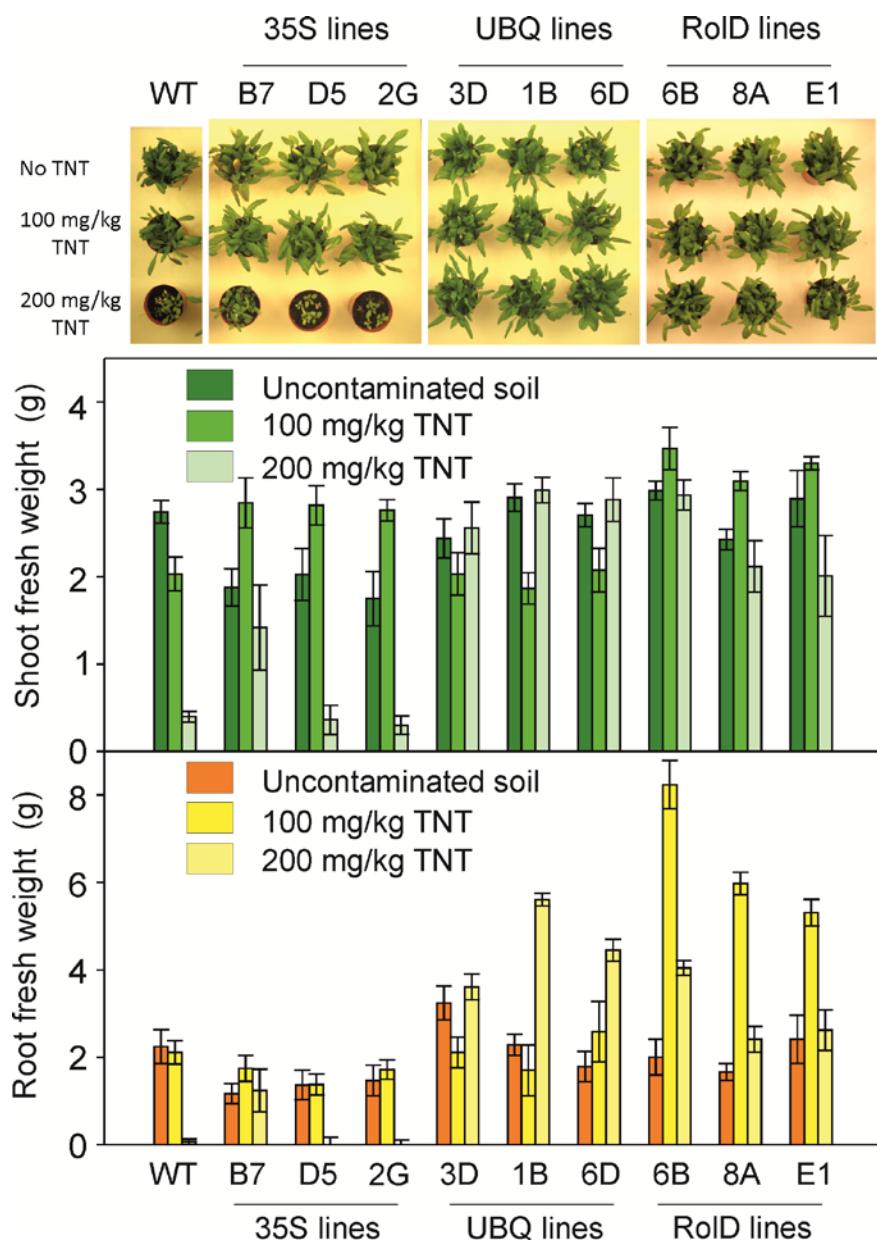
## 7.3 Results

Nine, independent homozygous Arabidopsis lines were produced, containing the *nfsI* gene under the control of the *UBQ* promoter or the root-specific promoter *RoLD*. We also used three, previously characterized, independent, homozygous 35S-*nfsI* lines. We tested the ability of these lines to remove TNT from liquid media. Our results showed that, after 24 hr, the unmodified plants had removed 39 % of the TNT. This is in agreement with previous results, and demonstrates the capacity of unmodified plants to detoxify and sequester this toxin. Of the transgenic lines, the *UBQ-nfsI* lines removed between 88 to 98 % of the TNT, the *RoLD-nfsI* removed 42 to 94 % and the 35S-*nfsI* lines 41 to 72 %. These results suggested that the *UBQ*-lines had the highest expression of active nitroreductase. However, these experiments were conducted in liquid culture, so the aerial parts of the plants were submerged, and could have taken up TNT. The aerial tissues of the *RoLD* lines lack the nitroreductase activity present in the constitutively-expressing 35S and *UBQ* lines.

Localization studies using <sup>14</sup>C-labelled TNT have shown that >99 % of the TNT taken up by plants is located in the roots, so in studies using TNT-treated soil, the *RoLD* lines may outperform the *UBQ-nfsI* lines. To test this hypothesis, lines containing *UBQ*, 35S and *RoLD* constructs were grown in untreated soil and soil treated with 100 mg/kg and 200 mg/kg TNT for six weeks and shoot and root biomass measured. For each promoter construct, the three lines that removed the highest level of TNT from liquid culture were selected for further work. The results (figure 7.1) showed that when plants were grown in untreated soil, there was no significant difference between the mean shoot weights of the *RoLD* transgenic lines and those of unmodified, wild-type plants. The 35S lines were apparently smaller than wild-type, which was unexpected and could possibly be explained by experimental variation. However, when grown in soil treated with 100 mg/kg TNT, the 35S- and *RoLD*-promoter lines had mean shoot biomasses 30 % and 60 % greater than wild-type, respectively. The shoot weights of the *UBQ*-promoter lines were not significantly different from wild-type. When the plants were grown in soil treated with 200 mg/kg TNT, the 35S-promoter lines had a 70 %

increase in shoot biomass compared with wild-type. The *UBQ*-promoter and *RolD*-promoter lines had much greater shoot biomasses than wild-type (700 % and 600 % greater than wild-type, respectively).

In the absence of TNT, the mean root weights of the *RolD*-promoter lines were not significantly different from wild-type. When grown on soil treated with 100 mg/kg TNT, the *RolD*-promoter lines, which expressed *nfsI* exclusively in the roots, had 300 % more root weight than the wild-type controls. The roots of the *35S*-promoter and *UBQ*-promoter lines had similar weights to wild-type. At the higher concentration of TNT (200 mg/kg), the root weights of the *UBQ*-promoter lines were not significantly different from those of the *RolD*-promoter lines, but were much greater than wild-type.



**Figure 7.1 Analysis of tissue-specific promoters expressing NR in Arabidopsis.**

The shoot and root biomass of Arabidopsis plants expressing the bacterial nitroreductase gene (*nfsI*) under the control of three different promoters (*35S*, *UBQ* and *RolD*) were measured. Plants were grown for seven weeks in a) untreated soil or b) soil treated with 100 mg/kg TNT. Results are means  $\pm$  SE of six replicate samples. WT, wild type.

In untreated soil, the ratio of shoot to root biomass was similar for all the plant lines. However, in the presence of TNT, the transgenic plants with increased ability to withstand the toxic effects of TNT allocated relatively more biomass to their roots than the wild-type plants did; as might be expected if the location in the roots of TNT and its breakdown intermediates is an important factor in the toxicity of TNT. Our studies following the detoxification of TNT by plants indicate that plants are unable to derive energetic value from TNT, so this cannot be the reason for the observed increased root biomass.

Overall, the *RolD*-promoter lines showed the most vigorous plant growth, so this promoter would be a candidate for transgene expression in field trials with dicot species. Within SERDP-ER-1498, we are also transforming monocot species (grasses) with *nfsI* and *xplA/B*, as described in Task 6. Promoters optimized for dicot expression do not necessarily give high expression in monocot species, hence the need to test a range of alternatives.

## Task 8

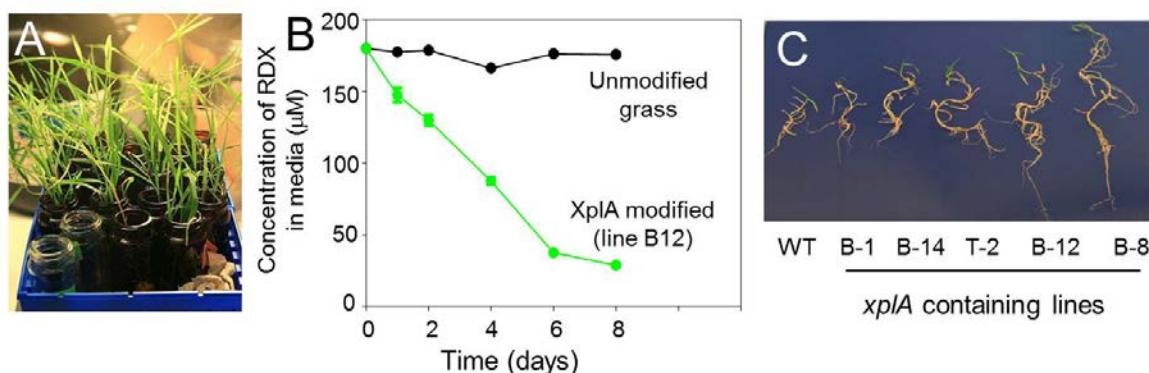
### Soil Studies with Transgenic Grasses

Experiments undertaken as part of Task 5 showed that *Arabidopsis* plants transformed with *nfsI*, *xplA* and *xplB* could take up and detoxify RDX from soil leachate. The purpose of the experiments in Task 8 was to demonstrate, at laboratory/glasshouse scale, that the transformed grasses were also capable of removing and degrading RDX from soil water. This property is clearly very important in phytoremediation applications on military ranges, since RDX is highly mobile in the soil environment and readily leaches into the water table.

#### 8.1 Experiments with creeping bentgrass

As described under Task 6, creeping bentgrass was successfully transformed with the target genes *nfsI* and *xplA*. Gene expression analysis using qPCR and western blots initially showed that *xplA* was transcribed and translated into protein.

Liquid culture studies at the University of Washington showed that *xplA*-expressing lines tolerated RDX toxicity and used RDX as a nitrogen source, with increased biomass that was positively correlated with *xplA* expression levels. The hydroponic studies demonstrated that *xplA*-expressing lines could remove significant quantities of RDX from solutions. The results are summarized in Figure 8.1.



**Figure 8.1 Summary of data on creeping bentgrass lines transformed with *xplA***

At the University of York, the capacity of transgenic creeping bentgrass to take up and degrade RDX from soil was tested, by watering soil-grown turf with water containing RDX. Creeping bentgrass lines transformed with *xplA* and *nfsI* were grown as turf in 50 cm wide x 70 cm long x 12 cm deep trays, along with unmodified and unplanted control trays. Each tray

was sequentially placed in a controlled environment chamber, with 18 °C day, 16 °C night temperatures and a 16 hr photoperiod. To measure RDX removal from soil leachate by these plants, each tray was saturated with water for 24 hr and then allowed to drain, with no further water added, for eight days. Following this, 14 litres of 160 µM RDX and 100 µM of the soil tracer dye sulforhodamine B were added uniformly across the soil surface. An initial 100 ml of soil leachate (time 0) were collected from the drainage holes, then these were resealed and the trays left to stand for 24 hr. Following this, the bungs were removed from the holes and the soil leachate collected. The trays were left to stand for a further 7 days, then flushed with 14 litres of uncontaminated water and the level of RDX measured in the collected soil leachate (time 7d). The results were analyzed by HPLC. As this was a preliminary experiment, single trays were set up for each of the four lines, alongside an unplanted control tray.

Analysis of the results showed that for the unplanted control tray, 52% of the RDX was recovered in the soil leachate, whereas for the xplA-expressing lines, the levels of RDX recovered were 8.8, 9.7 and 8.3 %, for lines B8, B12 and #14, respectively. These results were not significantly different from the 8.7 % recovered from the tray containing unmodified grasses, suggesting that the transgenic bentgrass lines were no better than wild-type at removing RDX from soil water. This may be due to problems with the experimental design, which may need optimizing. Alternatively, silencing of the xplA transgene may have occurred, reducing its expression. Western blots experiments subsequently confirmed this.

## 8.2 Experiments with switchgrass

Soil leachate studies were performed at the University of Washington, modelled on the protocols described in Jackson *et al.*, 2007. Switchgrass was grown with 16 hr photoperiod in Buchner funnels containing uncontaminated potting soil. Three replicate funnels were set up for each of the following treatments: soil only, wild-type switchgrass and transgenic switchgrass (N1 – see below), with ten plants per funnel. Each funnel was flooded with RDX solution, with a total mass of 1 mg RDX. For 6 weeks, the funnels were flushed weekly with water, collecting 100 ml of leachate for RDX analysis.

The mass of RDX collected in the leachates each week ranged from 0.024 mg to 0.051 mg for the funnels containing soil only, from 0.018 mg to 0.039 mg for wild-type switchgrass and from 0.013 mg to 0.036 mg for the transgenic switchgrass. The average total mass of RDX collected after 6 weeks was 0.224 mg for the soil-only funnels, 0.149 mg for the wild-type and 0.126 mg for the transformed switchgrass. The low recovery of RDX (approximately 22%) from the funnels containing soil only suggested that more RDX was bound to the potting soil than previously expected.

## Task 9

### Engineer XplA HMX-degrading *Arabidopsis* plant lines

A component of our research programme was the use of directed evolution to increase the activity of XplA towards RDX, and develop activity towards HMX (octogen), another explosive that can contaminate range land.

We developed a high-throughput Griess assay method and screened approximately 10,000 *xplA* mutants generated by error-prone PCR and *E.coli* mutator strains, but failed to identify any mutants with activity towards HMX. However, site-directed evolution produced three *xplA* mutants (M394L, M322L and V391A), with significant increases in HMX binding, compared to wild-type, although *in vitro* activity assays did not detect any degradation of HMX or release of nitrite or formaldehyde.

The structures of RDX and HMX are similar, although HMX molecules are larger. Using our knowledge gained from determining the structure of XplA, we predicted that to engineer

XplA to degrade HMX, we needed to increase the substrate channel for HMX to gain access to the active site. We used a rapid evolution approach on the heme pocket residues and one of the putative access channels of the mutant xplAs, with the aim of generating activity towards HMX. This strategy used the original Griess assay and a new colorimetric assay we developed to detect nitrite production. In this assay, colonies of *E.coli* transformed with the nitrite-sensitive promoter region *yeaR* upstream of the *lacZ* reporter gene in *pMOD-LacZ+yeaR* turn blue when grown in the presence of X-gal and 5 mM sodium nitrite. Screens of *xplA* variants have not yet identified any mutants with significant levels of HMX activity. It is conceivable that engineering HMX activity in an *xplA* variant requires more, strategic amino acid changes (>10) than are being generated using our techniques. A computational-based, rational design approach might be needed to bring about the significant structural changes required to confer activity towards HMX.

## Task 10

### Selection of explosive-transforming grasses

Progress towards the production of grasses engineered to detoxify RDX and TNT is described in detail in Tasks 6 and 8. A summary is provided here.

The work at the University of is being continued under ESTCP ER-201436.

#### 10.1 Switchgrass

Switchgrass was transformed with *xplA*, *xplB* and *nfsI*, using the new vector described in section 6.5. Hydroponic experiments at the University of Washington showed that the transgenic switchgrass can take up and degrade RDX from liquid media. Soil leachate studies were performed at the University of York, as described in Task 8, though the results were inconclusive. Demonstration-scale field experiments are in progress (Task 11). Switchgrass lines N1, N2 and N5 are being maintained in the glasshouse and growthroom, in preparation for planting in the field experiments.

Experiments were conducted to develop methods for seed production from our best RDX-degrading lines. Preliminary studies involved bagging the inflorescences of pot-grown plants, to promote self-pollination and seed set. No seeds were obtained from these plants, probably because of the known self-sterility of switchgrass. Also, the development of inflorescences was inhibited in the paper bags.

Subsequent experiments aimed to determine the ability of the switchgrass 'N' lines to cross-pollinate and produce viable seed. Ten plants each of lines N1 and N18 were grown in pots and cultured in the Biobubble pollen containment chamber at 25 °C, for three months. So far, all of the plants from the N1 and N18 lines have headed and are blooming. We have installed a fan in the Biobubble, to promote air movement and cross-pollination.

Experiments were also carried out with the putative self-compatible switchgrass line, NL94. The NL94 lines were cultured in two pots in the plant growth room, with shaking to promote self-pollination. However, only three seeds were obtained from 4 inflorescences and these experiments will be repeated.

#### 10.2 Creeping bentgrass

Tissue culture methods were optimized, as described for Task 6. A number of transformants was obtained, using the new vector with the monocot promoter, described in Section 6.5. Initial RDX uptake experiments showed that creeping bentgrass could take up and degrade RDX from liquid media (Section 6.6.1.3), though subsequent results suggested that this capacity may have been lost after repeated vegetative propagation of the creeping bentgrass lines. Soil leachate studies with creeping bentgrass turf appeared to show that, although the amount of RDX in soil leachate was not reduced by the transgenic grass, the amount of RDX

in the soil and in the plant tissues at the end of the experiment was lower than with wild-type turf (Task 8).

### **10.3 Western wheatgrass**

Tissue culture methods were optimized, as described for Task 6. Several western wheatgrass transformants were obtained, using a biolistic bombardment method, with the new vector described under Task 6.5.

### **Task 10.1**

#### **Increase rates of RDX degradation by transgenic western wheatgrass lines**

Transgenic lines of western wheatgrass were generated, as described in Task 6. They have not yet been tested for RDX degradation ability.

### **Task 10.2**

#### **Engineer transgenic wheatgrass to degrade RDX and detoxify TNT**

Progress towards the production of transgenic wheatgrass is presented in Task 6.

## **Task 11**

### **Perform glasshouse and field studies to validate the technology**

#### **11.1 Introduction**

The objective of this part of the project was to prepare for small-scale field trials, to demonstrate that the perennial grasses engineered to degrade explosives are capable of phytoremediating RDX in soils and reducing groundwater contamination in a field situation. Prior to actual field demonstration, transgenic and non-transgenic grasses were grown and evaluated for a variety of growth parameters under greenhouse conditions. Outdoor test cells, utilizing native and contaminated soils, were evaluated and modified, to characterize the uptake and degradation dynamics of RDX by transgenic grasses. In addition, all the required permits (USDA/APHIS/EIS) for transporting contaminated soils and using transgenic grasses in field demonstrations had to be completed.

#### **11.2 Greenhouse studies**

Several greenhouse studies were conducted to determine how to design and manage the proposed demonstration site. We constructed separate 28.4 m<sup>2</sup> boxes (117 x 244 x height 102 cm) in the greenhouse, to determine the correct spacing of western wheatgrass plants required to obtain suitable soil cover and to test the infiltration and recovery of soil water. The soil used was sand-based, free-draining and nutrient-poor; i.e. with similar physical and chemical properties to the soils in the field plots (Figure 11.1). Spacing the plants correctly was important, to allow the plants to spread vegetatively without excessive competition between them, while producing uniform cover allowing for maximum leaf or aerial growth and RDX uptake. The plants were separated either by 10 cm or 20 cm, in a grid pattern. The optimum spacing for production of root and leaf tissue was found to be 20 cm.

Three types of lysimeter were tested, for extraction of water samples to determine the quantity of RDX that remained in the soil substrate at any given time. Small volume (30 ml) lysimeters were found to be best for sampling soil depths less than 0.5 m, as the quantity of water available was limited.



**Figure 11.1 Western Wheatgrass transplants on 10 (background) and 20 (foreground) cm grid pattern**

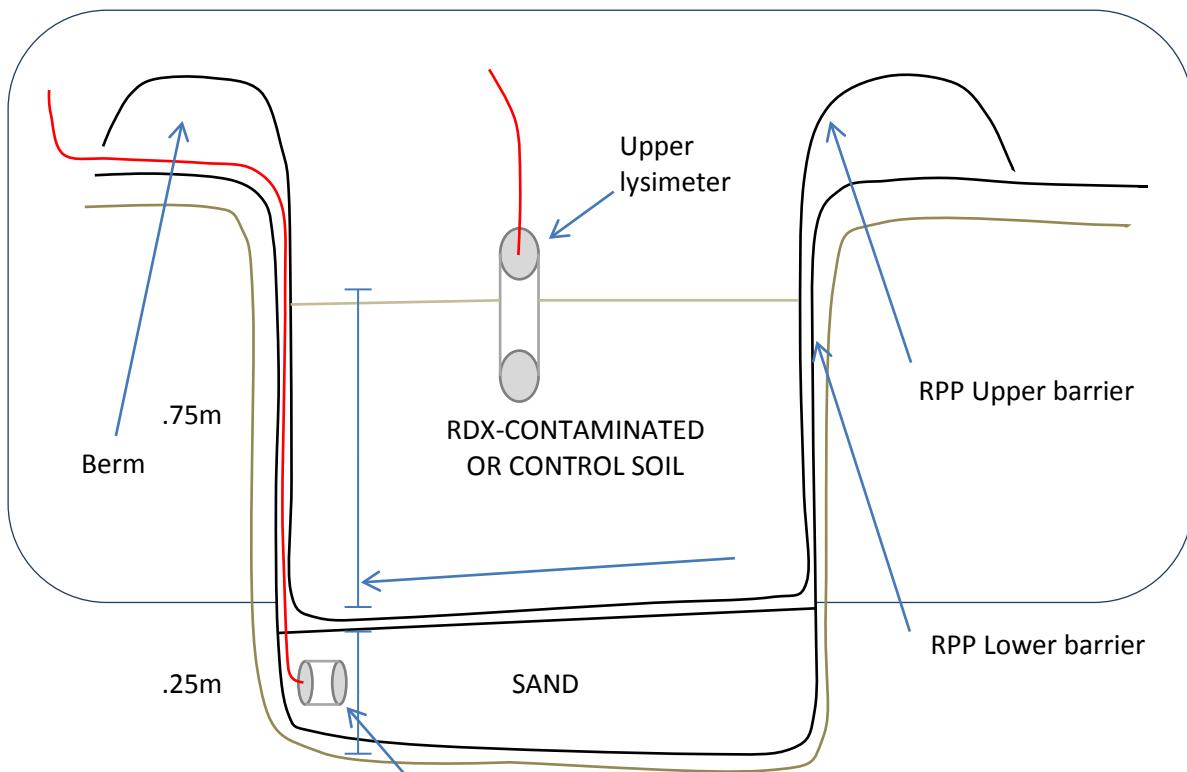
### **11.3 HPLC limits**

The RDX detection limit of the HPLC was tested. The lowest detectable RDX concentration was 50 ppb. To prepare for the analysis of water samples containing lower RDX levels than this, we performed solid-phase extraction (US EPA Method 3535A), to concentrate trace levels of RDX. RDX standards were prepared at 0.1 ppb, 1 ppb, 10 ppb and 100 ppb in 100 ml solutions. RDX was extracted from the solutions using a Porapak RDX cartridge, and then eluted from the cartridge with acetonitrile. The eluents were concentrated to 1 ml and analyzed by HPLC. The concentrations of RDX increased 100-fold. Hence, with solid-phase extraction, our detection limit for RDX is 1 ppb, which is at the regulatory level.

### **11.4 Outdoor field trials - Test cell construction**

Prior to implementing a full scale field demonstration, it was important to test construction methods and to evaluate containment procedures and the effects of a confined space on plant growth. Obviously, soil water dynamics will vary, due to seasonal and climatic factors, so also needed to be evaluated. Four test cells (3m x 3m x 0.75m) were constructed at CRREL (Figure 11.2). A liner of 60 mm-thick reinforced polyethylene was installed, 0.75 m below the existing soil surface. A 25 cm layer of clean silica sand was placed above the barrier and a suction lysimeter was installed in the sand layer, to monitor RDX leachate. A secondary liner was placed over the sand layer and refilled with a Charlataan silt loam soil. A second (upper) lysimeter was installed in the centre of the soil in each test cell, to measure soil water concentrations of nutrients and contaminants.

A drainage column was installed, to allow for the recycling of excess water. A drip irrigation system was also installed, to water the plants. Preliminary experiments using western wheatgrass showed that the containment system worked satisfactorily and the grass plants grew well.



**Figure 11.2** Above, two test cells under construction at CRREL. Below, cross section of the double-lined containment system that will be used as the transgenic plant field plots to be constructed at Fort Drum.

### 11.5 Demonstration site design and selection

As mentioned previously, the purpose of the demonstration site was to determine the extent to which transgenic grasses phytoremediate RDX under field conditions. The demonstration experiment was a randomized complete block design, set up with three soil treatments (1 ppm RDX, 100 ppm RDX and no RDX) and three vegetation treatments (untransformed grass, transformed grass and a no plant control), replicated three times for a total of 27 plots. Plots were 3 m x 3m square and 0.5 m deep, as illustrated in Figure 11.2. An additional 1 m x 3 m area was included in the containment plots to place the sump/irrigation holding tank within the containment area.

Each vegetation plot will be planted with approximately 250 grass clones, spaced on a 20 x 20 cm grid and allowed to establish for approximately one month, to obtain a sufficient root system. Plots receiving 1 ppm RDX would be dosed through the irrigation system. Plots receiving 100 ppm (i.e. 0.1 RDX g/kg soil) would be dosed with fine particulate RDX, mixed

thoroughly with the top 10 cm soil and irrigated with water, in order to simulate particulate munitions contamination around targets on training ranges.

The most important considerations when selecting a demonstration site are the availability of untreated and RDX-treated soil, and the availability of labour and equipment support. Environmental information about the site must be readily available, and the site should be near to ERDC-CRREL for monitoring at low cost.

Labour and equipment support from the Department of Public Works (DPW) and the Integrated Training Area Management (ITAM) offices were necessary to construct the demonstration site. Support from the Environmental Office was necessary to provide baseline data needed for completion of the USDA-APHIS permit requirements. The plants we have developed are intended primarily for training lands (storage, firing points and impact areas), but could also be used in areas where munitions are manufactured.

For the above reasons, Fort Drum NY was selected as our initial test location (Figures 11.3 & 11.4). The test plots were constructed using clean local soils and will be treated with RDX once the transplanted grasses are established. The sump/irrigation system has been installed and we are only awaiting APHIS approval on the permit.

## **11.6 Permitting Issues**

As part of our preliminary field research using test cells to evaluate soil water movement, we required RDX-contaminated soils which were well characterized and available in sufficient quantity to construct the 3m x 3m x 0.5m test cells. Hawthorne Army Ammunition Plant had soils meeting our requirements and we were granted a permit to transport these soils from Nevada to Vermont by the USDA. (However, as described in section 11.5, later experiments started with uncontaminated soil, with RDX added at the test concentrations.)

Initial briefings concerning our proposed timeline for implementation of small scale field testing at Ethan Allen Air Force Range were favourably received by the U.S. Department of Agriculture, Animal and Plant Health Inspection Service, USDA-APHIS. Obtaining the USDA-APHIS permit for conducting field experiments required that we complete an Environmental Impact Statement (EIS) for each proposed transgenic species. Ethan Allen Air Force Range personnel have been instrumental by assisting us in locating potential sites which have limited access and are well characterized from a soils (chemical, physical, and munitions constituents properties) and environmental perspective (plant/animal/insect/archaeology surveys). Our ability to locate several potential sites having this level of environmental detail made completion of an EIS relatively straightforward once we had provided information regarding the exact transgenic grass species we plan to utilize. At the time of writing (September 2015), we were still awaiting APHIS approval on the permit before the demonstration-scale experiments could go ahead.



**Figure 11.3 Location of demonstration plots**



**Figure 11.4 Experimental layout of RDX treatment plots**

## Task 12 Engineer biocontainment of transgenes

### 12.1 Introduction

Compared with transformation of the nuclear genome, plastid transformation has several advantages for biotechnology applications (Verma & Daniell, 2007). Plastid transgene expression can be remarkably high; for example, a desired recombinant protein may represent up to 70% of leaf protein (Ruhlman *et al.*, 2010). Also, chloroplast genes in most angiosperm plant species are maternally inherited, which means that transgenes in the chloroplast genome are not disseminated by pollen. This is clearly of benefit when considering biocontainment of transgenes that are to be introduced into the environment in plants for phytoremediation. It is an especially important advantage for regulatory approval of transgenic grasses, which produce copious, high mobility pollen that may promote hybridization with wild grasses in the field.

Another advantage of chloroplast transformation is that it is easier to construct vectors for transformation with multiple genes, due to the organization of the chloroplast genome into operons containing multiple genes under the control of a single promoter and terminator (Lu *et al.*, 2013).

The technique of chloroplast transformation of tobacco is well-established, hence we first attempted to demonstrate the activities of *xplA/B* and *nfsI* when those genes were inserted into the chloroplast of tobacco. If these efforts were successful, we planned to modify the vectors to target the chloroplast of grasses suitable for use on military training ranges.

## **12.2 Vector construction**

Details of the construction of the *pLZ* chloroplast expression vectors, target vectors (*pLZTNT*) and transformation vectors (*pLZTNT-AADA*) for tobacco chloroplast transformation are given in the Appendix (A12.1). An advantage of this vector system is that the promoters, 5'-UTRs and terminators in different *pLZ*s can be interchanged to produce a variety of modified *pLZ*s to enhance foreign gene expression. Another advantage is that expression cassettes containing foreign genes released from different *pLZ* vectors can be inserted into a single target vector. Thus the translation efficiency of every gene is enhanced, since every gene is flanked by a different 5'-UTR and a different terminator.

The marker genes *GUS*, *GFP* and *AADA* were constructed into separate *pLZ* vectors and expressed in *E.coli*, to confirm the function of the expression cassettes of the vectors. Experimental details are given in the Appendix (A12.2). Successful results with these markers demonstrated that our vector systems were suitable for the transformation of tobacco chloroplasts.

The RDX degradation genes *XplA* and *XplB*, the TNT detoxification gene *nfsI* and the selection marker gene *AADA* were amplified and inserted into the target vector *pLZTNT*, to produce the chloroplast transformation vector *pLZTNT-ABNR*. Further experimental details are provided in the Appendix.

In addition, a *pLD-xplA* vector was constructed as a control for our vector system. *pLD-ctv2* was a gift from Prof. Daniell at the University of Central Florida. This vector has previously been shown to work well for the expression of foreign genes in the chloroplast of tobacco. The *XplA* gene was inserted into the *pLD-ctv2* vector as a *NotI* fragment, to produce the *pLD-xplA* vector.

## **12.3 Chloroplast transformation in tobacco**

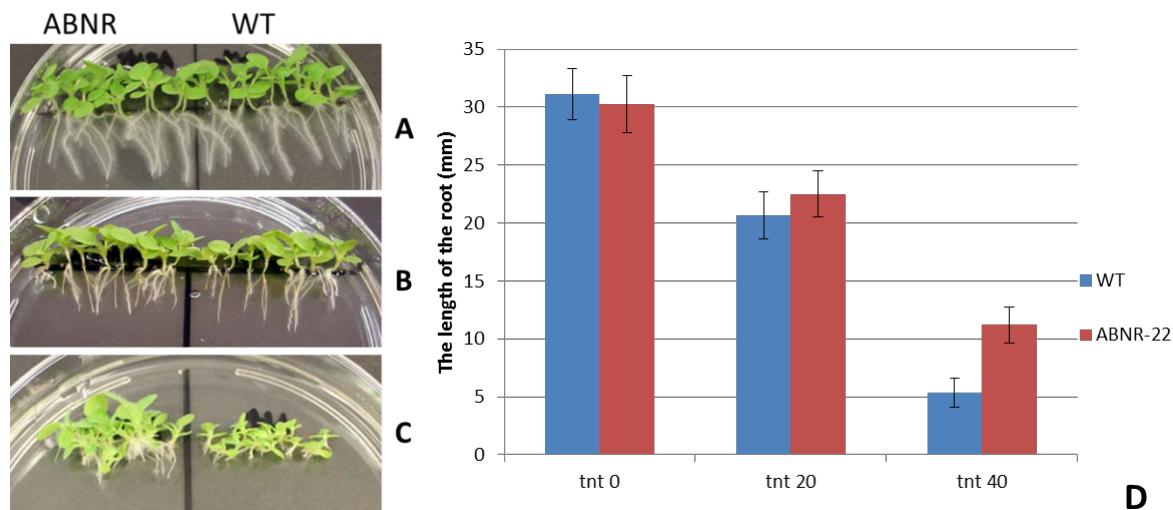
Plastid transformation was carried out as described in Verma and Daniell (2007), with some modifications. Briefly, one fully-expanded, dark-green leaf of tobacco (*Nicotiana tabacum*) was placed abaxial side up on RMOP medium (MS medium with 100 mg/l myo-inositol, 6-BAP at 1 mg/l and NAA at 0.1 mg/l). The transforming DNA was introduced by the biolistic process, using the biolistic device PDS1000/He and 0.6 µM gold particles (Bio-Rad, Hercules, CA). Bombarded leaf discs were placed on RMOT medium in darkness for two days. Leaf discs were observed under blue light fluorescent microscope and photographed using an LSM 5 PASCAL Laser Scanning Microscope. Two pieces of bombarded tobacco leaves (5 mm<sup>2</sup> each) were placed on RMOP selection medium with 500 mg/l spectinomycin, with the bombarded side in contact with the medium, for the first round of selection. Petri dishes were incubated in the culture room under white fluorescent lamps (1900 lux) with 16 hr light/ 8 hr dark photoperiod at 26 °C. After 4 to 8 weeks, putative transgenic shoots appeared. Before the second round of selection, 100 mg of leaf material were harvested from the putative transplastomic shoots. DNA was extracted, and PCR was used to confirm the integration of the foreign gene(s) in the chloroplast genome. For the second round of selection, 2 mm<sup>2</sup> pieces were cut from the leaves of plants that had tested positive in PCR, and placed on RMOP selection medium. These leaf sections produced transgenic shoots in 3-4 weeks. The regenerated shoots were excised and transferred to MS medium containing spectinomycin. This step was termed the third round of selection, in which rooting occurred after 3 to 4 weeks.

## **12.4 Molecular analysis of chloroplast transformed tobacco**

Molecular analysis of the transformed tobacco by PCR, RT-PCR and western blot showed that the *xplA/B* and *nfsI* genes were integrated into the chloroplast genome, and that the proteins accumulated in the chloroplast. Details are given in the Appendix (A12.7 & A12.8).

Total protein from wild-type tobacco and from wild-type Arabidopsis and Arabidopsis nuclear-transformed with *xplA-xplB-nfsI* was analysed in the same way, as controls. The results confirmed the accumulation of nfsI protein in the chloroplast-transformed tobacco.

**12.5 The effect of TNT on the growth of transplastomic tobacco, transformed with *nfsI*.**  
 RDX and TNT are normally used together in mixtures in military munitions, and since many plants are sensitive to TNT toxicity, it is necessary that transgenic plants engineered to degrade RDX must also be able to withstand TNT toxicity. *XplA-XplB-nfsI* transplastomic tobacco was cultured on solid or liquid MS medium dosed with TNT at different concentrations to test its response. Twenty seeds of wild-type and ABNR-transformed tobacco were sown on  $\frac{1}{2}$ MS medium dosed with TNT at 0  $\mu$ M, 20  $\mu$ M and 40  $\mu$ M and cultured in the light at 25 °C. The root lengths of the seedlings were measured and analyzed. The results showed that the roots of the transformed tobacco were significantly longer than wild-type after 21 days on solid medium with TNT at 40  $\mu$ M (Figure 12.1).



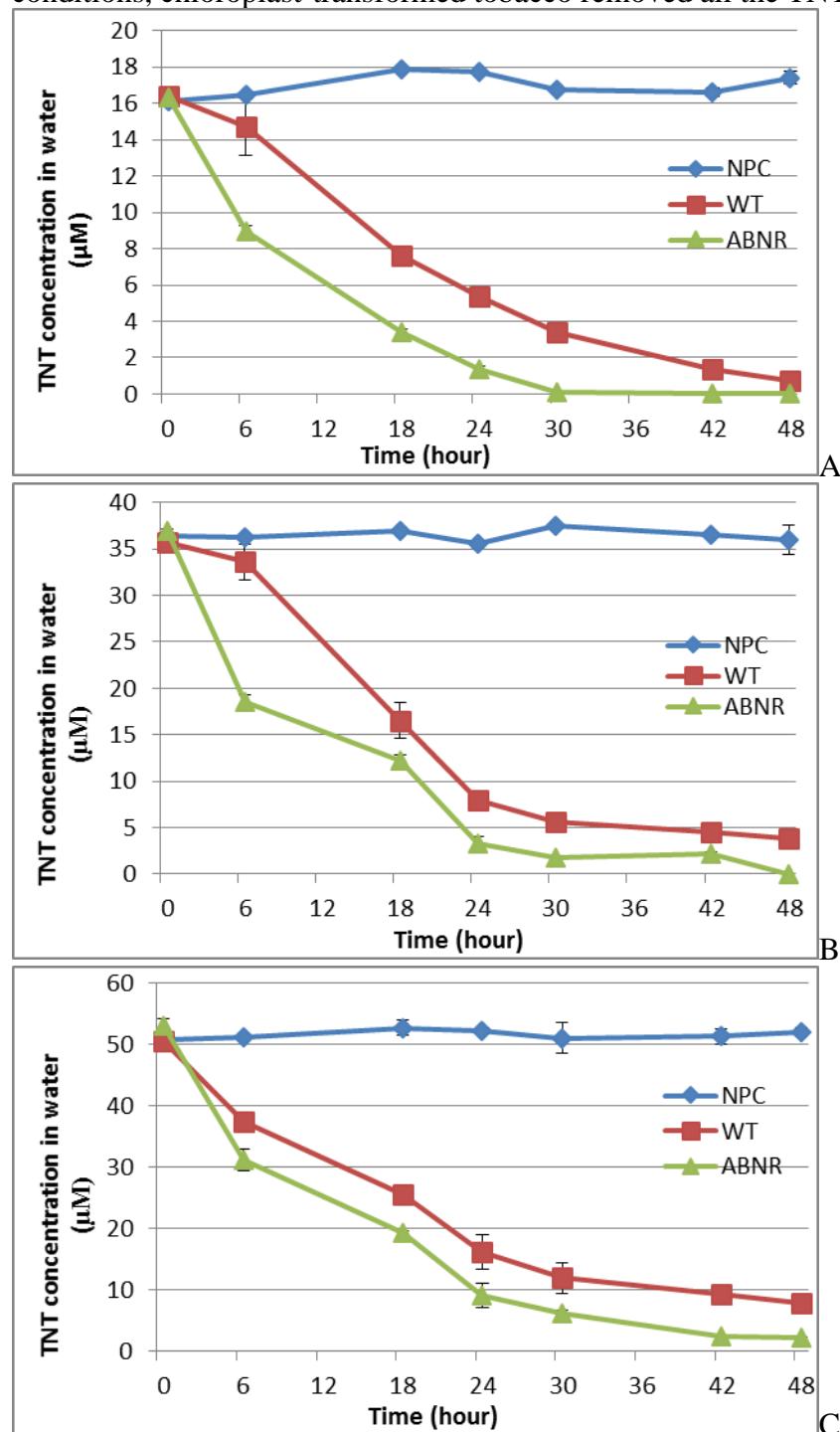
**Figure 12.1 The effect of TNT on root growth of chloroplast-transformed tobacco.**

To determine the effect of TNT on the biomass of transplastomic tobacco, wild-type and transformed tobacco seeds were sown on MS medium and cultured for 14 days. Seedlings were transferred to 125 ml flasks, with 20 ml modified liquid MS medium ( $\frac{1}{4}$  major +  $\frac{1}{2}$  minor + 0 Fe + 1% sucrose), dosed with TNT at different concentrations. Each flask contained 20 plantlets. The starting biomass and final biomass were recorded after 7 days culture. The results showed that in liquid medium, TNT depressed the growth of wild-type tobacco at concentrations below 20  $\mu$ M, and the transformed tobacco accumulated more biomass compared to wild-type at different TNT concentrations.

## 12.6 TNT uptake by transplastomic tobacco

To determine TNT uptake by chloroplast-transformed tobacco, seeds of wild-type tobacco and transformant abnr26 were initially sown on MS medium or MS medium with spectinomycin at 500 mg/l and cultured at 25°C with a 16 hour/ 8 hour dark photocycle. Twenty 15-day-old plantlets were transferred to 125 ml flasks with 20 ml ddH<sub>2</sub>O dosed with TNT at 0, 20, 40 and 60  $\mu$ M. Each bottle contained 20 plantlets, comprising about 0.4 g tobacco biomass. The medium was sampled at intervals of several hours and the TNT concentration determined by HPLC analysis.

The results showed that both wild-type and transformed tobacco could efficiently remove TNT from the water (Figure 12.2). When the plants were exposed to 20  $\mu$ M TNT, all the TNT in the water was removed by the transformed tobacco after 30 hours' culture, while the wild-type tobacco needed 48 hours to remove all the TNT. For the 40  $\mu$ M and 60  $\mu$ M treatments, the removal of TNT by wild-type tobacco decreased after several hours of exposure to TNT and the TNT concentration plateaued at 5  $\mu$ M and 10  $\mu$ M, respectively. Under the same conditions, chloroplast-transformed tobacco removed all the TNT from the water.



**Figure 12.2 TNT uptake by chloroplast-transformed tobacco.**

Twenty 15-day-old plantlets of ABNR chloroplast-transformed tobacco or wild-type tobacco were cultured in 20 ml water dosed with TNT at (A) 20  $\mu$ M, (B) 40  $\mu$ M or (C) 60  $\mu$ M.

## **12.7 RDX experiments with transplastomic tobacco**

Tobacco transformed with *pLZTNT-ABNR* or *pLD-xplA* was cultured in liquid ½MS medium, amended with 180 µM RDX. The concentration of RDX in the medium was determined, by HPLC analysis, after 2, 5 and 7 days' culture. Three replicate samples were taken. Wild-type tobacco was used as a negative control and Arabidopsis nuclear genome-transformed with *xplA-xplB-nfsI* was used as a positive control. After 7 days' culture, RDX was extracted from the plant samples and analyzed, as follows. Leaf samples (500 mg) were placed in 2 ml tubes with stainless steel balls and ruptured by shaking in the Fast Prep machine. 1 ml methanol was added per tube, and the tubes vortexed. The tubes were shaken at 100 rpm overnight and centrifuged at 15000 g for 10 min. The supernatant (900 µl) was pipetted to a new tube and centrifuged again for 10 min, prior to transfer of 800 µl of supernatant to a 1 ml amber vial for HPLC analysis.

The results showed that there was no significant difference in RDX uptake from the medium for *xplA-xplB-nfsI* transformed tobacco (or *pLD-xplA* transformed tobacco) and wild-type tobacco. However, RDX in the medium of the positive control, Arabidopsis nuclear-transformed with *xplA-xplB-nfsI*, decreased rapidly to zero. Analysis of the RDX concentrations in the leaves showed that there was no significant reduction in the RDX concentration of the chloroplast-transformed tobacco, compared with wild-type (data not shown). Hence, the chloroplast-transformed tobacco did not apparently degrade RDX. The nuclear-transformed Arabidopsis, however, contained no detectable RDX; the result expected if the positive control was working.

## **12.8 Construction of a new vector for enhancing the expression level of *xplA* in chloroplasts**

As discussed previously, tobacco chloroplast transformed with *pLZTNT-ABNR* was unable to degrade RDX compared to wild-type, even though western blot against *xplA* protein was positive. We speculated that the concentration of *xplA* protein in the chloroplasts was too low to degrade RDX efficiently. The expression cassette for *xplA* was modified to enhance the translation efficiency. The *xplA* gene was inserted into *pLZ1* with the *psba* promoter and terminator. In addition, a ribosome binding site was added at the 5' end of the *xplA* gene. As a control to monitor the expression level, a *GFP* gene was inserted into the vector, with a separate *psba* promoter and 5' ribosome binding site. The revised combination of promoter and 5'-UTR was tested in *E.coli* with the visible reporter protein *GFP*. We also added a short ribosome binding sequence, GGAGG, at the 5' end of the *GFP* gene, and inserted the *GFP* gene into the *pLZ* vector as an *AgeI-MluI* fragment. *E.coli* harbouring this construction produced a strong GFP signal. The *xplA* gene was linked with the GGAGG sequence and inserted into the *AgeI-NsiI* site of *pLZ1*, to produce *pLZ1-xplA*. The expression cassette of *xplA* was released as an *AscI* fragment and inserted into the *pLZTNT-5aada* chloroplast transformation vector, to produce the stacked *pLZTNT-1xplA-5aada* vector. By the end of this project, we had transformed tobacco with the new chloroplast transformation vector by gene gun bombardment, and obtained three tobacco lines that were PCR positive for *xplA*.

# **Task 13**

## **Develop methods for seeding grasses on ranges**

### **13.1 Introduction**

Remote impact areas are inaccessible to standard tillage and seeding equipment, so establishing plants to control soil erosion in these areas presents unique challenges. Clearly, nutrients, moisture and good seed/soil contact are necessary for plant establishment, and previous experiments where seed has been broadcast aerially have not been successful.

Seedballs provide a reservoir of nutrients and water and are a potential method to increase establishment success. Seedballs have traditionally been made by incorporating seeds into compost, clay or manure, and are designed to sow seeds without tilling, especially on thin, compacted soils.

## 13.2 Objectives and background

The primary objective of our demonstration at West Point was to seed a portion of the impact area from the air, using a helicopter. As discussed at our meeting on 17th December 2009, the major problem in the impact area was found to be soil erosion caused by loss of plant material related to the use of 105 mm HE shells and 60 and 81 mm mortars shot over a period of about six weeks (June to mid-July 2009). The primary target area was approximately 300 to 400 m wide, with the entire damaged area being about 1.5 acres in size. The area lacked sufficient vegetative cover to control soil erosion, and soil was eroding from the slope and settling at the slope base. The area is only used for 2 months of the year, so the vegetation at the site has 10 months to recover. The soil at the site is a glacial moraine, on a north-facing slope. The soil is generally acidic, and the eroded soil is fine-textured.

The desired vegetation for seeding should be fire resistant and drought tolerant. Aerial seeding using seedballs was proposed as a solution to the inaccessibility of the site. Experiments to optimize the formulation and application of the seedballs were carried out for different grass species.

## 13.3 Seedball experiments

### 13.3.1 Initial seedball formulation studies

Experiments were conducted at the CRREL glasshouse, to evaluate the use of seedballs as a means of grass seed propagation. Our early trials focused on a mixture of potting soil and clay, but the seedballs broke down rapidly and would not survive the drop from a helicopter. Tests involving xanthan gum (a food thickener) began in March 2010. Initial trials evaluated xanthan gum concentrations, seed concentrations and the effects of a 14 foot drop on the integrity of the seedballs and the germination of the seeds. Optimum gum concentration, based on successful trials in Hawaii, was found to be 0.65% (w/w). Gum concentration levels below 0.65% produced seedballs that lacked sufficient cohesion to maintain structural integrity. Seed germination was not dependent on the concentration of xanthan gum.

Trials at higher xanthan gum concentrations produced seedballs with greater structural integrity that were easier to form into a uniform sphere, approximately the size of a golf ball. Hence the gum concentration was raised, to 1.3%.

### 13.3.2 Fourteen foot seedball drop

Seedballs dropped from a height of 14 feet remained intact and the seeds germinated. Deformation of dropped seedballs depended on both moisture content and gum concentration. When saturated seedballs were dropped onto a hard surface, they flattened out, which provided a larger soil/seedball interface.

### 13.3.3 Seed concentration for seedballs

Initially, the seed concentration of perennial ryegrass (*Lolium perenne L.*) was approximately 50 seeds per seedball. 12 seeds germinated after two weeks. Within one month, seedballs were covered in dense growth of perennial ryegrass. In addition to the dropped seedballs, undropped seedballs were placed on the sand. The tray of seedballs was then placed in the greenhouse and watered daily. Dropped and undropped seedballs produced lush growth of perennial ryegrass, indicating that, under moist conditions, it was not critical for seedballs to

have good soil contact for plant germination and growth. In later trials, the concentration of perennial ryegrass seeds was reduced to approximately 25 seeds per ball.

#### **13.3.4 Dehydration process for seedballs**

Dehydration experiments were conducted to investigate seed viability after a drying process. If seeds remained viable after dehydration, seedball production could take place in a lab or glasshouse, prior to distribution in the field.

Newly-made seedballs were dehydrated by air-drying (in a growth chamber at 28°C) for two days, to a consistent moisture content. In the glasshouse, daily watering rehydrated the seedballs, and 8 seeds per ball germinated after 3 weeks.

Trials of larger batches of seedballs also demonstrated the capacity of the seedballs to withstand dehydration while maintaining seed viability. When the amounts of potting soil needed were greater than 10 kg, an electric cement mixer was used for mixing. This provided the capacity to make batches of approximately 1500 seedballs at one time. Seedballs were dehydrated in a growth chamber at 28 °C for two days and germination tests were carried out. Dehydrated seedballs did not provide reliable rapid germination.

#### **13.3.5 Fifty foot seedball drop**

A drop test was conducted at a greater height (50 feet), using re-hydrated seedballs at four moisture content levels (dry; partially-wet (5 ml); moist (10 ml); saturated). The seedballs were dropped onto sand. The dry seedballs remained intact or split in half. The partially-wet seedballs remained whole, split or shattered. The moist and saturated seedballs spread out on impact.

### **13.4 Summary**

Seedball experiments at CRREL demonstrate their potential use for seeding inaccessible sites. Dehydration of seedballs did not prevent future germination of perennial rye-grass. Xanthan gum was a readily available cohesive agent and had no detectable negative effect on the germination or growth of perennial rye-grass. Rehydration of seedballs before sowing was required to improve germination. Slow-release fertilizer pellets could be added to the seedball mixture to improve grass growth.

## **Task 14**

### **Monitor insect herbivory on genetically modified plants**

#### **14.1 Introduction**

An important question to address is whether the introduction of the *xplA*, *xplB* and *nfsI* transgenes into plants adversely affects herbivores that may ingest the plants.

Investigating the likely environmental impact of these transgenic plants on herbivory and food chains provides important data contributing towards ecological risk assessments, regulatory approval and public acceptance.

We conducted studies to test whether an insect herbivore (locust; *Schistocerca gregaria*) favours or avoids eating Arabidopsis plants grown in RDX-treated soil. We also compared herbivory on Arabidopsis plants expressing *xplA* and *xplB* with wild-type, unmodified plants. Locusts are generalist grass-feeding insect folivores and are used as a model insect system in herbivory assays.

#### **14.2 Materials and Methods**

Arabidopsis plants were grown either in untreated soil, in soil treated with a low level of RDX (100 mg/kg) or soil treated with a high level of RDX (1000 mg/kg) for five weeks. Herbivory was compared between the following combinations of genotype and soil treatment:

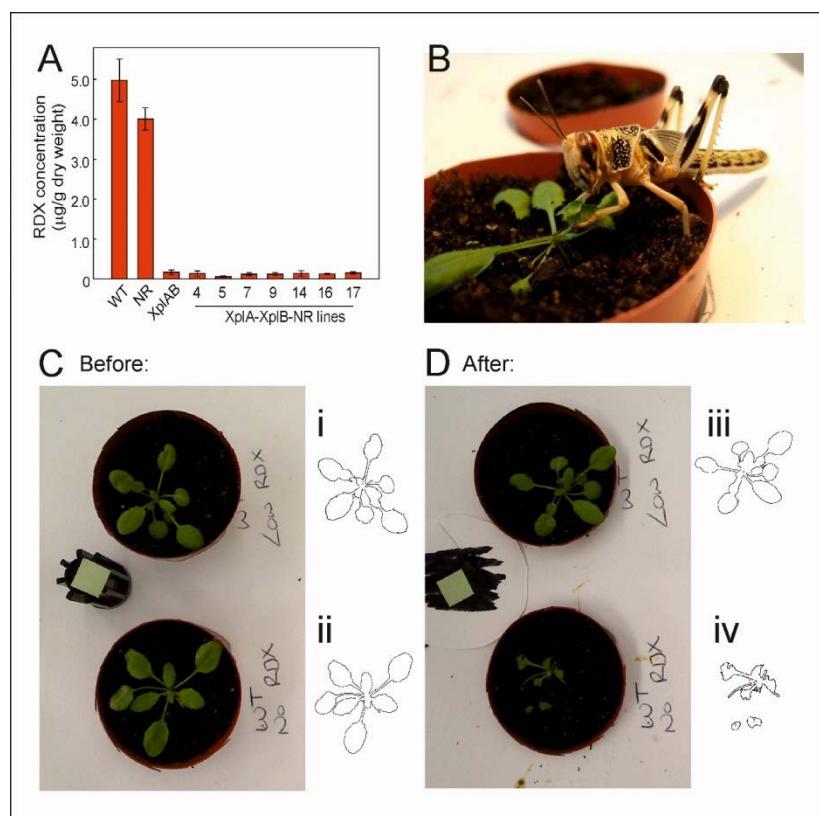
Wild-type; No RDX  
Wild-type; No RDX  
Wild-type; Low RDX  
Wild-type; No RDX  
Wild-type; Low RDX  
Wild-type; High RDX  
XplA/B; No RDX  
XplA/B; No RDX  
XplA/B; Low RDX

compared with  
compared with

Wild-type; Low RDX  
Wild-type; High RDX  
Wild-type; High RDX  
XplA/B; No RDX  
XplA/B; Low RDX  
XplA/B; High RDX  
XplA/B; Low RDX  
XplA/B; High RDX  
XplA/B; High RDX

Experiments were performed with paired plants, with 11 replicates for each group.

Individual, 4th instar locust nymphs were allowed to choose between two equally-sized plants in a choice chamber (figure 14.1) and monitored until approximately 50% of the foliage of one plant had been eaten. The plants were photographed before and after herbivory, and the surface areas determined using ImageJ software.



**Figure 14.1 . Herbivory preference trials.**

(A) Plants expressing XplA/B activities accumulate significantly less RDX in the aerial tissues than unmodified plants grown on RDX-contaminated soil. (B) The generalist insect herbivore locust *Schistocerca gregaria* feeding on Arabidopsis. Appearance of plants (C) before and (D) after feeding preference trial. (i, ii, iii and iv), surface area profiles from photographs before and after feeding were used to determine herbivory.

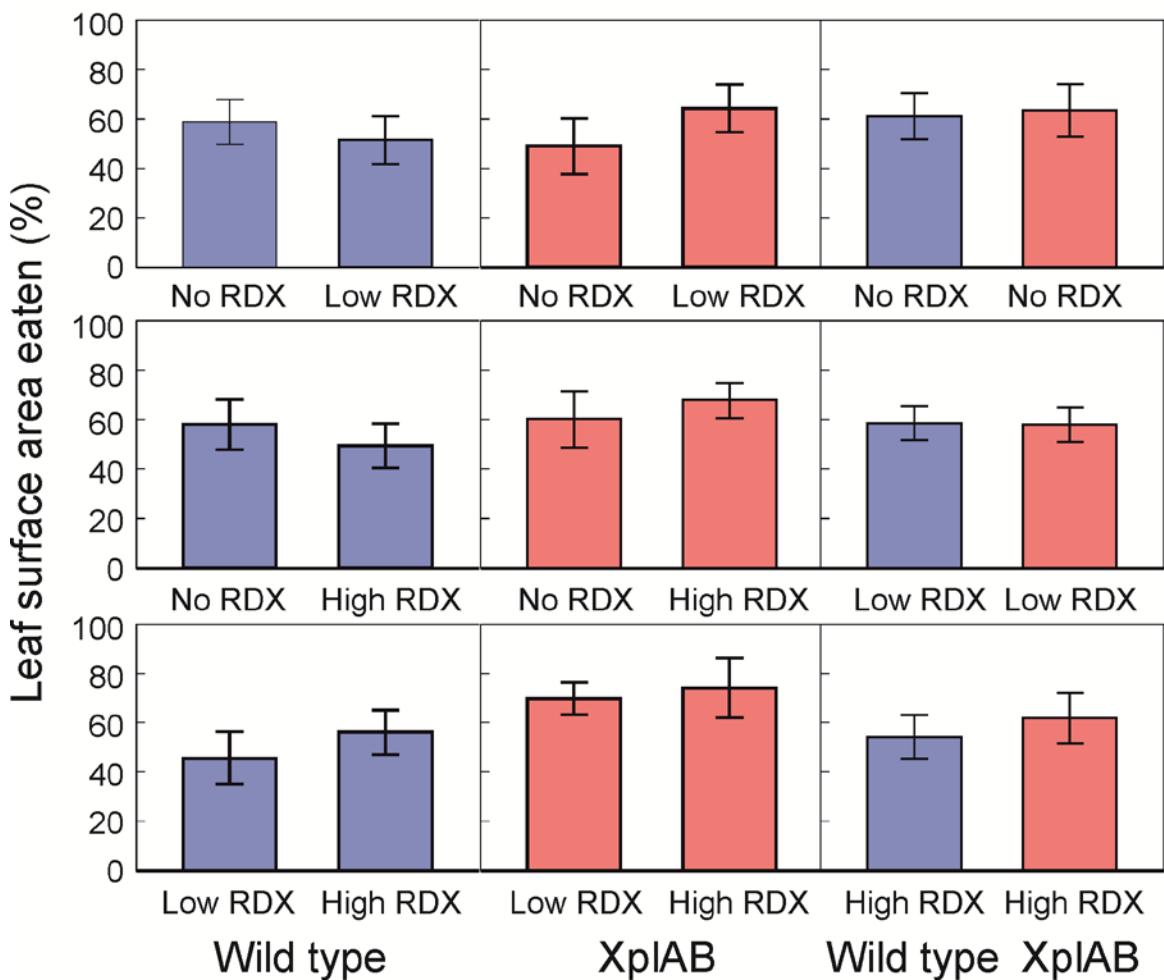
### 14.3 Results

Analysis of the data showed that the locusts did not exhibit a preference for either plant in any of the above combinations (Figure 14.2).

Our previous work has shown that plants expressing XplA/B accumulate significantly less RDX in their aerial tissues than unmodified (wild-type) plants, when grown on RDX-treated

soil (Rylott *et al.*, 2011b). XplA/B plants also produced higher biomass than wild-type plants on RDX-treated soil.

XplA activity degrades RDX, to produce nitrite and formaldehyde and the ring degradation products NDAB and MEDINA. While these products are detectable *in vitro*, they have not been found in planta and it is likely that they are metabolized by endogenous plant enzymes, so XplA/B plants would be predicted to have high nitrogen levels. Insect herbivores are known to prefer, and seek out, plants with higher nitrogen levels, thus our results are encouraging as they suggest that XplA/B plants will not be specifically targeted (or avoided) by herbivores when growing in the field.



**Figure 14.2 Results of *Arabidopsis* herbivory preference tests.**

Results are mean +/- se of 12 replicate pairings for each comparison.

### Task 15

#### Assess the impact of genetically modified plants on soil microflora

One of the overall aims of phytoremediation is to decrease the toxicity of the contaminated soil, to allow the indigenous microflora to flourish and contribute to the remediation process. Soil bacteria play an essential role in many ecosystem processes, including the decomposition of organic matter. Thus, a decrease in microbial biomass and diversity, resulting from exposure to toxic chemicals such as TNT, negatively affects soil function and fertility. It is

therefore important to determine the impact of transgenic grasses on the ecotoxicology of soil contaminated with TNT and RDX. We aimed to investigate whether transgenic grass plants could positively influence the functional and genetic diversity of the microbial communities in contaminated soil.

Once the transgenic grass lines with RDX- and TNT-degrading activity had been characterized, we intended to carry out genetic analysis of the bacteria in bulk soil and the rhizosphere, by direct pyrosequencing of cDNA-RNA tags from the metagenome. However, the preparation of the transgenic grass lines was problematic and took much longer than expected. Therefore, there was insufficient time to perform soil microflora studies with the genetically modified grasses.

As part of SERDP ER-1318, we had previously carried out experiments to investigate the impact of transgenic tobacco on microbial communities in soil contaminated with TNT. Our results showed that in uncontaminated soil, expression of NR by tobacco plants did not have a non-target effect on soil microflora. In the presence of TNT, the rhizosphere community of the NR-expressing plants sustained higher numbers of culturable bacteria than wild-type, with greater functional and genetic diversity. The results are presented in detail in Travis *et al.*, (2007).

## Conclusions and Implications for Future Research

We have demonstrated that our technology works in the model dicot species *Arabidopsis*, and that even within this relatively small plant, the expression of our RDX-degrading, TNT-detoxifying transgenes *xplA/B* and *nfsI* confers sufficient activity to remove saturating levels of RDX from soil leachate. We have overcome considerable technical difficulties to show that this technology can be successfully transferred to monocot species, such as creeping bentgrass, western wheatgrass and switchgrass. We developed a new transformation vector system, using monocot promoters, in order to achieve this. Switchgrass transformed with *xplA/B* and *nfsI* is now ready for demonstration-scale field trials.

Aside from this, we have identified additional proteins that are involved in TNT-tolerance in *Arabidopsis*, viz. GTases, OPRs, GSTs and cytochromes P450. The knowledge obtained from these studies could provide a platform from which to develop future phytoremediation strategies. In addition, the demonstration of the function of MDHAR6 in TNT-toxicity could have wider implications for the development of novel herbicides.

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 Siberian wheatgrass [http://plants.usda.gov/factsheet/pdf/fs\\_agfr.pdf](http://plants.usda.gov/factsheet/pdf/fs_agfr.pdf)  
 Creeping bentgrass <http://www.fs.fed.us/database/feis/plants/graminoid/agrsto/all.html>  
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## Appendices

### A. Supporting Information - protocols and experimental details

#### Task 5

##### A5.1 Protocol for plasmid construction and transformation of *Arabidopsis* with *xplA*, *xplB* and *nfsI*.

The *xplA* gene was cloned into the binary vector *pMLBART* (Gleave, 1992), which confers resistance to the herbicide Basta, to produce the vector *pMLBART-xplA*. The *xplB* gene was cloned into *pART27* (Gleave, 1992), which confers resistance to kanamycin, to produce the vector *pART27-xplB*. The *nfsI* gene, encoding the nitroreductase activity, was cloned into *pART27* (Gleave, 1992) to create *pNITRED3* (Hannink *et al.*, 2001), and separately into *pJ0530*, a *pBIN19* derivative (Bevan, 1984) which confers resistance to hygromycin, to create *pJ0530-nfsI*. All constructs expressed *xplA*, *xplB* and *nfsI* under the control of the CaMV35S promoter and *ocs* terminator. Approximately fifty, independent *Arabidopsis* transformants were generated (in the Col-0 background) for each construct, using *Agrobacterium*-mediated floral dipping. Independent, homozygous lines expressing *xplA* were as characterized previously (Rylott *et al.*, 2006). Three to five independent homozygous lines containing the *xplB* or *nfsI* constructs were used for further study. These lines were selected because they had segregation ratios indicative of single insertion events and relatively high rates of removal of RDX or TNT from liquid culture (Rylott, *et al.*, 2006; Jackson *et al.*, 2007). XplA-NR plants were generated by crossing the best *pNITRED3 NR*-expressing line (B7) with the best *XplA*-expressing line (line 10). The triple XplA-XplB-NR lines were generated firstly by transforming line 35S::*XplA10* (Rylott *et al.*, 2006) with the *pART27-xplB* construct. The XplAB lines with the highest rates of removal of RDX from liquid culture (lines 2 and 27) (Jackson *et al.*, 2007) were re-transformed with *pJ0530-nfsI*, to generate XplA-XplB-NR lines. The presence of the transgenes was confirmed in all lines by PCR (data not shown). Seven, XplAB-derived, homozygous lines, independently transformed with *nfsI* were selected for further analyses, based on the criteria outlined above. A summary of the plant lines used in this study is presented in Table A5.1.

Plant line	Parent line(s)	Construct(s)	Marker genes			Transgene present			Source
			bar	hptII	nptII	nfsI	xplA	xplB	
Wild-type Col-0	-	-	-	-	-	-	-	-	NASC (line N1094)
35S:XplA-10	-	pMLBart- <i>xplA</i>	yes				yes		Rylott <i>et al.</i> 2006
B7	-	pNITRED			yes	yes			
2G	-	pNITRED			yes	yes			
D2	-	pNITRED			yes	yes			
XplA-NR	35S:XplA-10 B7	pMLBart- <i>xplA</i> pNITRED	yes		yes	yes	yes		
XplAB-2	35S:XplA-10	pMLBart- <i>xplA</i> <i>pART27-xplB</i>	yes		yes		yes	yes	Jackson <i>et al.</i> 2007
XplA-XplB-NR	XplAB-2	pMLBart- <i>xplA</i> <i>pART27-xplB</i> <i>pJ0530-nfsI</i>	yes	yes	yes	yes	yes	yes	

**Table A5.1. Summary of plant lines, binary vectors and transgenes.**

##### A5.2 Transgene expression analysis

Rosette leaves of six-week-old, soil-grown plants were harvested and ground in liquid nitrogen. For transcript analysis, mRNA was extracted individually from five plants per line

using an RNeasy kit (Qiagen) and treated with DNase (Qiagen) to remove genomic DNA. 1 µg of total RNA was then used to synthesize cDNA, using oligo (dT) 12-18 primers (Invitrogen) and SuperScript III Reverse Transcriptase (Invitrogen). Quantitative RT-PCR was performed with an ABI7300 real-time PCR detection system, using SYBR green (Bio-Rad). Primer sequences were; forward 5' TACAGTGTCTGGATCGGTGGTT 3' and reverse 5' CGGCCTTGGAGATCCACAT 3' for ACTIN2; forward 5' CGACGAGGAGGACATGAGATG 3' and reverse 5' GCAGTCGCCTATACCAGGGATA 3' for *xplA*, forward 5' CACCGCAATCGGTTCG 3' and reverse 5' GTACAGGCCCGGAGCAAGA 3' for *xplB* and forward 5' ACACGCCGGAAGCCAAA 3' and reverse 5' GGTGCATGTCGGCGAAGTA 3' for *nfsI*. Relative expression values were calculated using ACTIN mRNA as an internal reference.

For western analysis, 10 µg of crude protein extract from rosette leaves were loaded per lane. Antibodies to the XplA protein, as used in Rylott *et al.*, (2006), and NR protein were raised in rabbit. A goat, anti-rabbit alkaline phosphatase-conjugate was used as secondary antibody.

### A5.3 Complementation of *mdhar6-1*

The following primers were used to amplify the mitochondrially targeted form of MDHAR6, At1g63940.2, from cDNA, using the PCR conditions and method described for MDHAR6.1 recombinant expression. The sequence was cloned into *KpnI* and *BamHI* restriction sites of *pART7* and then binary vector *pART27*: MDAR6.2 *KpnI* 5'-ggtaccATGTCTGCAGTTCTCGAG-3' MDAR6.2 *BamHI* 5'-ggatccCTAACTCTGTAGAGCG-3'. The *pART27-mito-MDHAR6* construct was introduced into Col-0 and *mdhar6-1* plants, using *Agrobacterium*-mediated floral dipping.

### A5.4 Recombinant expression of MDHAR6 in *E.coli*.

The MDHAR6.1 transcript was amplified from cDNA derived from 14-day old Arabidopsis seedlings using the PCR, with a high-fidelity *Pfu* polymerase and using the following primer sequences:

MDAR6.1BamH1	ggatccATGGCGTTAGCATC	MDAR6.1SalI
gtcgacCTAACTCTGTAGAGCGG.	The PCR parameters were 95 °C for 2 min, then 35 cycles of 95 °C, 30 sec; 50 °C, 25 sec; 74 °C, 3 min, followed by a 5 min step at 74 °C. The PCR product was cloned into <i>pET16b</i> and sequence verified.	

#### A5.4.1 *E.coli* competent cell transformation

Aliquots of *BL21 DE3* *E. coli* competent cells containing 0.2% (v/v) 2-mercaptoethanol were transformed with either *pET16b:MDHAR6* or empty *pET16b* vector as a negative control, via 30 min incubation on ice, 20 sec (Arctic Express DE3) or 45 sec (BL21 DE3) 42°C heat pulse, and further 2 min incubation on ice. The cells were incubated in SOC medium for 1 hr at 37 °C with 250 rpm shaking, and plated on LA containing 50 µg/ml carbenicillin.

#### A5.4.2 Expression of MDHAR6 in *E.coli* competent cells

Individual transformed colonies were inoculated into 40 ml of LB containing 50 µg/ml carbenicillin, and incubated at 37 °C with 250 rpm shaking overnight. An 11.25 ml aliquot of starter culture was then inoculated into 450 ml M9 media (9.93 mM Na<sub>2</sub>HPO<sub>4</sub>, 4.58 mM KH<sub>2</sub>PO<sub>4</sub>, 1.78 mM NaCl, 3.89 mM glucose, 0.92 mM MgSO<sub>4</sub>, 46.2 µM CaCl<sub>2</sub>) containing 50 µg/ml carbenicillin. Once culture OD600 approximated 0.6, expression of MDHAR6 was induced by addition of IPTG to a final concentration of 1 mM, with incubation at 20 °C and 250 rpm shaking for 3 hr. Cultures were then centrifuged at 5,000 rpm (15 min, 4 °C), and pellets resuspended in 10 ml of His-binding buffer (50 mM sodium phosphate pH 8, 300 mM NaCl) then sonicated with 0.2 mM PMSF at amplitude 70 for 4 min (3 sec on, 7 sec off cycles). Samples were then centrifuged at 15,000 rpm (15 min, 4 °C) to remove debris, then incubated with 1.75 ml pre-washed HIS-Select Nickel Affinity gel (Sigma-Aldrich), with rocking at 6 °C for 1 hr, and then at room temperature for a further 1hr 30 min. The resin was packed by centrifugation at 2,000 rpm, then washed three times with 26.25 ml His-binding

buffer, using 5 min centrifugation at 2,000 rpm (4 °C) to repack resin between washes. Non-specifically bound protein was eluted by washing with 1.75 ml Wash Buffer (50 mM sodium phosphate pH8, 300 mM NaCl, 5 mM imidazole), and centrifuging at 2,000 rpm (5 min, 4 °C), and then target protein eluted by washing in 1.75 ml Elution Buffer (50 mM sodium phosphate pH8, 300 mM NaCl, 300 mM imidazole) for 30 min at 6 °C with rocking. The resin was removed by centrifugation at 13,000 rpm (5 min) then via filtration through a 0.22 µm spin filter (Aligent Technologies 5185-5990). The filtrate was then dialysed within a 10K Dialysis Casette (ThermoScientific 87730) against 5 L of Dialysis Buffer (50 mM potassium phosphate, pH 7, 6 °C) for 14 hr 30 min, and then a fresh 5 L of Dialysis Buffer for a further 2hr 15 min.

#### A5.4.3 MDHAR activity assay

MDHAR activity of the His-tag purified and non-purified soluble fractions was assayed using a protocol modified from Colville & Smirnoff (2008); 625 µl of 50 mM TRIS buffer (pH 7.6, 25 °C) containing 1.0 mM EDTA, 25 µl of 4.29 mM NADH, 50 µl of 2.5 mM sodium ascorbate and 200 µl of sample were mixed by inversion in a cuvette. Absorbance at 340 nm (correlating with NADH concentration) was followed for >2 min before and >2 min after the addition of 100 µl of 5.6 U/ml ascorbate oxidase (Sigma) in 4 mM sodium phosphate buffer with 0.05% BSA pH 5.6.

#### A5.5 Expression of recombinant *Arabidopsis* GSTs in *E.coli*

GSTs were amplified from cDNA derived from 14-day old *Arabidopsis* seedlings exposed to TNT. The GSTs were then cloned into the BseRI site of pET-YSBLIC3C. Reactions using T4 polymerase were performed in 40 µl volumes containing; 0.5 pmol vector DNA, 4 µl 10x T4 polymerase buffer, 6.25 mM dTTP, 12.5 mM DTT and 2.5 units T4 DNA polymerase (Merck). T4 polymerase reactions with the insert contained 0.2 pmol of PCR product, 2 µl 10 x buffer, 2.5 mM dATP, 5 mM DTT and 1 unit T4 DNA polymerase (Merck). Both reactions were incubated at 22 °C for 30 min and then polymerase-inactivated by heating at 75 °C for 20 min. The vector reaction was then purified using a Wizard extraction kit (Promega) and DNA content quantified. To anneal, 2 µl of insert and 1 µl vector DNA were incubated for 10 min at room temperature. The reaction was stopped by the addition of 1 µl 25 mM EDTA for a further incubation for 10 min at room temperature. The product was then transformed into *E. coli* and positive clones identified by colony PCR. Cultures of *E. coli* expression host BL21DE3 containing *pET-YSBLIC3C-GST* were grown in 1 litre of ZY medium (10 g tryptone, 5 g yeast extract in 1 litre water) in a 2 L flask containing autoinduction additives and 30 µg/ml kanamycin, at 37 °C with 180 rpm shaking to O.D.600 of 0.8 – 1.0. Cultures were then transferred to a 20 °C incubator with 180 rpm shaking. Expression time varied from 24 h to 72 hr. Cultures were harvested by centrifugation at 8500 x g and the pellets weighed and re-suspended to 0.3 g/ml in cold PBS, pH 7.4. Cells were then lysed by sonication prior to purification. Cell lysates were centrifuged at 17,500 x g, 4 °C for 30 min to remove the cell debris. The supernatants were passed through a 0.45 µM syringe filter then purified using glutathione Sepharose 4B beads (Amersham Bioscience). A 1.33 ml stock of beads was washed three times in 10 ml cold PBS by centrifuging at 500 x g and removing supernatant between each wash. The beads were then re-suspended in 1 ml PBS. The filtered cell supernatant was applied to the beads and incubated for 30 min at room temperature with gentle shaking. After incubation, the beads were centrifuged at 500 x g at 4 °C for 5 min, the supernatant removed and the beads washed three times in cold PBS. To elute, beads were re-suspended in GST-elution buffer (10 mM reduced glutathione, 100 mM Tris pH 8.0, 120 mM NaCl) and incubated at room temperature for 15 min with gentle shaking. The purified enzyme was collected in the supernatant following centrifugation, 500 x g at 4°C for 5 min. SDS-PAGE gels were run to evaluate the purification and visualize if any enzyme had been lost during the wash steps. The purified protein concentration was determined with Pierce

Coomassie Dye Binding Reagent, with 1 ml reagent and 1-10 µl purified protein. The change in absorbance at 595 nm was measured with reference BSA standards of known protein levels to calculate the purified protein concentration. The identities of the purified proteins were confirmed by N-terminal sequencing.

#### A5.5.1 GST assay conditions

To determine how much TNT substrate to use in the assays, a concentration assay using a range between 1 – 200 µM TNT (concentrations well within the aqueous solubility limit for TNT of 500 µM) was used. The assay was performed in 100 mM potassium phosphate buffer pH 6.5 with 5 mM glutathione, at 30 °C and stopped by addition of 10% v/v TCA (240 mg/ml). All assays were performed in triplicate and analyzed using HPLC. The mobile phase gradient conditions (v/v) were 80 % 2 mM ammonium acetate, 20 % methanol for 4 min, then 40 % 2 mM ammonium acetate, 60 % methanol over 3 min, a constant 40 % 2 mM ammonium acetate, 60 % methanol for 5 min, then 80 % 2 mM ammonium acetate, 20 % methanol over 3 min and finally a constant 80 % 2 mM ammonium acetate, 20 % methanol for 10 min. Total run time was 25 min at a flow rate of 1 ml/min. The retention times for TNT and the GST-TNT conjugate were 12.3 and 5.8 min respectively. Peak areas were integrated using Empower software according to the manufacturer's instructions.

#### A5.6 Overexpression of GSTs in Arabidopsis

The *pART7/27* binary vector system (Gleave, 1992) was used to clone GSTs under the control of the CaMV35S promoter and *ocs* terminator, for near-constitutive expression in Arabidopsis of GST U1, U3, U4, U7, U22, U24 and 25. Primer design was facilitated using Primer3 design ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)). Arabidopsis cDNA from TNT treated plants was used as a template from which the GSTs were amplified. PCR was performed with Phusion High-Fidelity DNA polymerase (NEB). Primers are listed in Table A5.2 and the PCR program is shown in Table A5.3.

The TOPO cloning protocol (Invitrogen) was employed to clone the GST PCR products into the *pCR2.1-TOPO* vector. GST inserts were excised from TOPO with the restriction sites shown in Table A5.2. They were cloned into *pART7* which houses a cassette containing a CaMV-35S promoter, multiple cloning site and *ocs* terminator, flanked by *NotI* restriction sites. This cassette containing the gene was cut from *pART7* with *NotI* and ligated into *pART27*.

Primer	Restriction site	Sequence
35S GSTU1F	<i>EcoRI</i>	<u>GAA</u> TTC ATG GCG GAG AAA GAA GAG AG
35S GSTU1R	<i>BamHI</i>	<u>GGA</u> TCC TTA GGC AGA CTT AAT TGT C
35S GSTU3F	<i>EcoRI</i>	<u>GAA</u> TTC ATG GCC GAG AAA GAA GAG G
35S GSTU3R	<i>BamHI</i>	<u>GGA</u> TCC TTA GAC CGC TTT GAT TC
35S GSTU4F	<i>EcoRI</i>	<u>GAA</u> TTC ATG GCG GAG AAA GAA GAG G
35S GSTU4R	<i>BamHI</i>	<u>GGA</u> TCC TTA GGC TGA TTT GAT TC
35S GSTU7F	<i>EcoRI</i>	<u>GAA</u> TTC ATG GCG GAG AGA TCA A
35S GSTU7R	<i>BamHI</i>	<u>GGA</u> TCC TCA AGC AGA TTT GAT ATT G
35S GSTU22F	<i>EcoRI</i>	<u>GAA</u> TTC ATG GCG GAT GAA GTG
35S GSTU22R	<i>BamHI</i>	<u>GGA</u> TCC TTA GAC ACA GTA TAT CTT CC
35S GSTU25F	<i>EcoRI</i>	<u>GAA</u> TTC ATG GCA GAC GAG GTGA
35S GSTU25R	<i>BamHI</i>	<u>GGA</u> TCC CTA TTC GAT TTC GAT CC
35S GSTU24F	<i>KpnI</i>	<u>GGT</u> ACC ATG GCA GAT GAG GTG ATT CTT
35S GSTU24R	<i>XbaI</i>	TCT AGA TTA CTC CAA CCC AAG TTT GTT

Table A5.2 Primers for amplification of GSTs from *Arabidopsis* cDNA.

Step	Temperature	Time	
Initial denaturing	98 °C	30 s	
Denaturing	98 °C	10 s	30 cycles
Annealing	60 °C	30 s	
Extension	72 °C	30 s	
Final extension	72 °C	5 min	

**Table A5.3 PCR cycle conditions for the amplification of GSTs from *Arabidopsis* cDNA**

## Task 6

### A6.1 Primers used in transformation of grasses

Primer name	sequence	Restriction site flanked	Apply to
<i>HptF3</i>	TTGAATTCTATTATGAAAAAGCCTGAACTC	<i>EcoRI</i>	Clone and Insert <i>hpt</i> gene into <i>pSAT1a-35S</i> to produce <i>pSAT35S-hpt</i>
<i>HptR3</i>	ATTGGATCCCTATTTCTTGC	<i>BamHI</i>	
<i>XplaF5</i>	TTAAGCTTACCATGGCCGACGTAACGTGCCTG	<i>HindIII</i>	Clone and insert <i>XplA</i> gene into <i>pSAT6a</i> and <i>pNSAT6a</i>
<i>XplaR5</i>	TTAAGCTTCAGGACAGGACGATCGGC	<i>HindIII</i>	
<i>XplbF1</i>	ACGGTACCATGGACATCATGAGTGAAGT	<i>KpnI</i>	Insert <i>XplB</i> into <i>pSAT4a</i> and <i>pNSAT3a</i>
<i>XplbR1</i>	TTGGATCCTCAGCAGACCGATTGGGCCGGC	<i>BamHI</i>	
<i>NrF1</i>	TCGAATTCAACAATGGATATCATTCTGTCG	<i>EcoRI</i>	Insert <i>nfsI</i> gene into <i>pSAT7a</i> and <i>pSAT4a</i>
<i>NrR1</i>	TTGGATCCTCAGCACTCGGTACAATCG	<i>BamHI</i>	
<i>OsActinF1</i>	TTACCGGTCTCGAGGTATTAT	<i>AgeI</i>	Clone and insert <i>Osactin</i> promoter into <i>pSAT1a</i> to produce <i>pNSAT1a</i>
<i>OsActinR1</i>	TTAAGCTTCTACCTACAAAAAGCTCC	<i>HindIII</i>	
<i>ZmUbiF1</i>	TTACCGGTGCAGTGCAGCGTG	<i>AgeI</i>	Clone and insert <i>ZmUbi</i> promoter into <i>pSAT3a</i> to produce <i>pNSAT3a</i>
<i>ZmUbiR1</i>	CCAAGCTTGCAGAACGAAACACC	<i>HindIII</i>	
<i>PvUbiF1</i>	TTACCGGTCCACTGGAGAGGG	<i>AgeI</i>	Clone and insert <i>PvUbi</i> promoter into <i>pSAT6a</i> to produce <i>pNSAT6a</i>
<i>PvUbiR1</i>	TTAAGCTTGATCTGCATCTGCAGAAG	<i>HindIII</i>	
<i>GFPF5</i>	TTAAGCTTATTATGGTAGATCTGACTAGT	<i>HindIII</i>	Clone and insert <i>GFP</i> gene into <i>pNSAT1a</i>
<i>GFP5</i>	ATTCTGCAGTCACACGTGGTGGTGG	<i>PstI</i>	

<i>GUSF4</i>	TTGGAATTCAATTATGGTAGATCTG	<i>EcoRI</i>	Clone and insert <i>GUS</i> gene into <i>pNSAT3a</i> and <i>pNSAT6a</i>
<i>GUSR4</i>	TTAGGATCCTCACACGTGGTG	<i>BamHI</i>	
<i>Sat4seqf1</i>	CGAATCTCAAGCAATCAAGC		PCR to confirm the insertion of <i>hpt</i> cassette of <i>pRCS2-ABNR-HR</i> vector in grass genome DNA.
<i>Sat4seqr1</i>	CCTTATCTGGGAACTACTCAC		
<i>Nsat1aseqf1</i>	GCTGCTTCGTCAGGCTTAGAT		Work Together with <i>sat4aseqr1</i> to do PCR to confirm the insertion of <i>hpt</i> cassette of <i>pRCS2-NABNR</i> vector in grass genome DNA.
<i>Nsat3aseqf1</i>	CTTGATATACTGGATGATGGC		PCR to confirm the insertion of <i>xplB</i> cassette of <i>pRCS2-NABNR</i> vector in grass genome DNA.
<i>Sat3aseqr1</i>	AGCCACGCACATTAGGA		
<i>Nsat6aseqf1</i>	TGCTGTGATGCTGTTGTTG		PCR to confirm the insertion of <i>xplA</i> cassette of <i>pRCS2-NABNR</i> vector in grass genome DNA.
<i>Sat6aseqr1</i>	CCGGAAACAAACAACGA		
<i>Xpla300f</i>	CAACAACCGCGATCGACATCC		RT-PCR of <i>xplA</i> gene
<i>Xpla959r</i>	TCGAACATCGCCTCCATCAC		
<i>XplB511F</i>	GAATTCACCGGTTCCGATCT		RT-PCR of <i>xplB</i> gene
<i>XplB1143R</i>	GACTGTCCGGTCGATCACTT		
<i>Nfs1322f</i>	TTCAACACGCCAGAACCCA		RT-PCR of <i>nfsI</i> gene
<i>Nfs1652r</i>	AGCACTCGGTACAATCGT		
<i>HptF2</i>	TCTTAGCCAGACGAGCGGGTTC		RT-PCR of <i>hpt</i> gene
<i>HptR2</i>	TACTTCTACACAGCCATGGTCCAG		

**Table A6.1 Primers used in transformation of grasses**

## Task 12

### A12.1 Construction of expression vectors *pLZ*

Sequences for promoters, 5'UTRs, and terminators for the regulation of gene expression and translation in chloroplast of tobacco were cloned by PCR (Accuprime Taq DNA polymerase high fidelity, Invitrogen). These DNA fragments were constructed together to produce 5 expression vectors, referred to as *pLZ1* through *pLZ5*. The DNA fragments of the promoters were flanked by *EcoRI*, an 8 base rare cutting recognition site, and a *XbaI* site at the 5' end and a *KpnI* site at the 3' end. The 5'UTR fragments were flanked with *KpnI* and *BamHI* sites.

The terminators fragments were flanked with a *Pst*I site at the 5' end and a *Sph*I site, an 8 base rare cutting recognition site and a *Hind*III site at the 3' end. The promoters, 5'UTRs, terminators, and the primers used to amplify the promoters, 5'UTRs, and terminators are listed in Tables A12.1 and A12.2

Each promoter fragment was cloned into the *Eco*RI-*Kpn*I site of *pUC19* to produce *pUC19-p*. The vector *pUC19-p* was digested with *Eco*RI, then treated with DNA polymerase I, large (Klenow) fragment (Biolab) to fill-in 5' overhangs forming blunt ends, and to eliminate the *Eco*RI site, then producing *pUC19-p-e-k* by self-ligation. Each 5'UTR was cloned into the *Kpn*I- *Bam*HI sites of *pUC19-p-e-k* to produce *pUC19-p-5utr*. Each terminator fragment was cloned into the *Pst*I- *Hind*III site of *pUC19-p-5utr* to produce *pUC19-p-5utr-t*. The *pUC19-p-5utr-t* vector was digested with *Bam*HI and *Pst*I and ligated with a *Bam*HI-*Age*I-*Eco*RV-*Mlu*I-*Xho*I-*Nsi*I-*Pst*I linker to produce *pLZ*. Five *pLZ* vectors, referred to as *pLZ1*, *pLZ2*, *pLZ3*, *pLZ4*, and *pLZ5* were produced, containing different combinations of promoter, 5'UTR, and terminator.

<b>vector</b>	<b>promoter</b>	<b>5'UTR</b>	<b>terminator</b>	<b>8 base site</b>
<b>pLZ1</b>	<i>psba</i>	<i>Psba</i>	<i>psba</i>	<i>AsISI</i>
<b>pLZ2</b>	<i>rrn</i>	<i>rbcL</i>	<i>rbcL</i>	<i>AscI</i>
<b>pLZ3</b>	<i>rbcL</i>	<i>rbcL</i>	<i>rbcL</i>	<i>FseI</i>
<b>pLZ4</b>	<i>rrn</i>	<i>atpb</i>	<i>rps16</i>	<i>NotI</i>
<b>pLZ5</b>	<i>rrn</i>	<i>T7 gene 10</i>	<i>petD</i> (3'UTR)	<i>PmeI</i>

**Table A12.1. Elements for construction of chloroplast expression vectors *pLZ1-5*.**

Abbreviation: *psba*, photosystem II protein D1; *rrn*, ribosomal RNA operon; *rbcL*, ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit ; *atpb*, ATP synthase CF1 beta subunit; *rps16*, ribosomal protein S16; *T7 gene 10*, The T7 phage gene 10; *petD*, cytochrome b6/f complex subunit IV.

#### A12.2 Expression of *GUS*, *GFP* and *aada* genes in *pLZ* for expression cassette function test in *E.coli*.

In order to provide a timely confirmation of our ability to transform the chloroplast, vectors were constructed for transient expression of the visual markers GUS and GFP, and transformed into the tobacco plants.

The cDNA sequence of *GUS* was amplified with *pCAMBIA1301* as template (with the intron deleted) and cloned into the *Bam*HI-*Pst*I site of the *pLZ* vector to produce *pLZ-GUS*. Similarly, *GFP* was amplified from *pCAMBIA1302* and cloned into the *Bam*HI-*Mlu*I site of the *pLZ* vector to produce *pLZ-GFP*. The selection marker for all of our chloroplast transformation procedures was the *aada* gene, a selection marker gene that confers spectinomycin resistance for function in chloroplast transformed tobacco. The *aada* gene was amplified from *pLD-ctv2* (kindly provided by Professor Daniel of the University of Central Florida) and cloned into the *Bam*HI-*Mlu*I site of *pLZ* to produce the vector *pLZ-aada*. The DH5 $\alpha$  strains harbouring *pLZ-GUS* were cultured on solid LB medium with ampicillin at 100 mg/L and x-gluc at 2 mM and cultured at 37C overnight. The DH5 $\alpha$  strains harboring *pLZ-GFP* were cultured on solid LB medium with ampicillin at 100 mg/L and cultured at 37C overnight. The DH5 $\alpha$  strains harboring *pLZ-aada* were cultured in liquid LB medium with ampicillin at 100 mg/L and spectinomycin at 100 mg/L and cultured at 37C with shaking overnight. The GFP signal was observed under UV light.

Primers for promoter amplification	PL	Ppsbaf2	ATGAATTCCCGCATCGCTCTAGAGGTTGAATCCCGGG
	Z1	Ppsbar2	GCGCGGTACCATTCAACAGTATAACATGAC
	PL	Prrnf2	ATGAATTCCGGCGCGCCTCTAGAGAGTTTAGTTGG
	Z2	PrrnR2	TACGGTACCGTATCCATGCGCTTCATATTCTG
	PL	PrbcF1	AAGAATTCCGCCGCCCTCTAGACTGTCAAGGGGGAAAG
	Z3	PrbcR1	GGCCGGTACCTGCCAAATACATCATTATTG
	PL	PrrnF3	ATGAATTCCGGCCGCTCTAGAGAGTTTAGTTGG
	Z4	PrrnR2	TACGGTACCGTATCCATGCGCTTCATATTCTG
	PL	PrrnF5	ATCGAATTGTTAAACTCTAGAGAGTTTAGTTGG
	Z5	PrrnR2	TACGGTACCGTATCCATGCGCTTCATATTCTG
Primers for 5'UTRs amplification	PL	Lpsbaf1	ATGGTACCAACAAGCCTCCATTTCATT
	Z1	LpsbaR1	ATTGGATCCGGTAAAATCTGGTTATTAATC
	PL	LrbcF1	TCAGGTACCTCGAGTAGACCTTGTGTTG
	Z2	LrbcR1	ATTGGATCCAATCCCTCCCTACAACTC
	PL	LrbcF1	TCAGGTACCTCGAGTAGACCTTGTGTTG
	Z3	LrbcR1	ATTGGATCCAATCCCTCCCTACAACTC
	PL	LatpBF1	GAGAGGTACCATTCCATAGAAATAGATAATATG
Primers for terminator amplification	Z4	LatpBR1	GTAAGATCCAATAATAAAATAATATGTCG
	PL	Z5	The L t7gene 10 was synthesized by Operon company, the full sequence is as follows: gacGGTACCTAGAAATAATTGTTAACCTTAAGAAGGAGATATAC GGATCCttc
Primers for terminator amplification	PL	Tpsbaf2	GCGCCTGCAGATCCGGCCTACTCTATAGG
	Z1	TpsbaR2	ATAAGCTTGCATCGCGATGCCGAATATAGCTCTTC
	PL	TrbcF1	GGCGCCTGCAGAAACAGTAGACATTAGCAG
	Z2	TrbcR2	TTAAGCTTGGCGCGCCGCATGCTAGATTTGTATTTC
	PL	TrbcF1	GGCGCCTGCAGAAACAGTAGACATTAGCAG
	Z3	TrbcR3	TTAAGCTTGGCGCGCCGCATGCTAGATTTGTATTTC
	PL	Trps16F1	GCGCCTGCAGTCAACCGAAATTCAATTAAGG
	Z4	Trps16R1	ATAAGCTTGCAGCCGCATGCAGAACACGGAATT
	PL	TpetDF1	GGCCCTGCAGATTAAATTGATTCAATTGTG
	Z5	TpetDR2	TGACAAGCTTAAACGCATGCTAATATCATATATTC

**Table A12.2. Primers for amplification of DNA fragments for vector construction**

### A12.3 Transient expression of GFP gene in chloroplast of tobacco

Plastid transformation was carried out as described in Verma and Daniel (2007), with some modifications. Briefly, one fully expanded, dark-green leaf of tobacco (*Nicotiana tabacum*) was placed abaxial side up on RMOP medium (MS medium with 100 mg/L myo-inositol, 6-BAP at 1 mg/L and NAA at 0.1 mg/L) and the transforming DNA was introduced by the biolistic process using the biolistic device PDS1000/He and 0.6 µm gold particles (Bio-Rad, Hercules, CA). Two days after bombardment, leaf discs were observed under blue light fluorescent microscope and photographed with a LSM 5 PASCAL Laser Scanning Microscope.

### A12.4 Construction of target vectors and transformation vectors for tobacco chloroplast transformation

The upstream flanking region including the full length of 16s rDNA and *trnI* was amplified using the chloroplast genome of tobacco as a template and inserted into the *pGEM5Zf* vector as a *AatII-EcoRV* fragment to produce the *pGEM-up* vector. The downstream flanking region

including the full length of *trnA* was amplified and inserted into *pGEM-up* as a *Sall*-*NdeI* fragment to produce the vector *pGEM-up-down*. An *EcoRV*-*AsiSI*-*Ascl*-*FseI*-*NotI*-*PmeI*-*Sall* linker was then inserted into the *pGEM-up-down* vector to produce the target vector *pLZTNT*. There are five, 8 base-pair restriction sites in the target vector for insertion of up to five expression cassettes released from *pLZ1-pLZ5*. The expression cassette of the *aada* gene was released from *pLZ* with the corresponding 8-base restriction enzyme and inserted into *pLZTNT* separately, to produce the transformation vector *pLZTNT-aada* (1-5). The five vector variants of *pLZTNT-aada* were used to bombard leaf discs of tobacco for insertion of the *aada* gene into the tobacco chloroplast genome.

The RDX degradation gene *xplA* was amplified and inserted into the *pLZ2* vector as a *AgeI*-*NsiI* fragment. The *xplB* gene was inserted into the *pLZ3* vector as a *BamHI*-*MluI* fragment. The TNT degradation gene *nfsI* was inserted into the *pLZ4* vector as a *BamHI*-*MluI* fragment, and the selection marker gene *aada* was inserted into the *pLZ5* vector as a *BamHI*-*MluI* fragment. The expression cassettes of *xplA*, *xplB*, *nfsI*, and *aada* were cut from the *pLZ* vector with *Ascl*, *FseI*, *NotI*, *PmeI*, respectively, and successively inserted into the target vector *pLZTNT* to produce the transformation vector *pLZTNT-2xplA-3xplB-4nfsI-5aada*, which henceforth is referred to as *pLZTNT-ABNR*. The *pLZTNT-ABNR* vector was used to bombard leaf discs of tobacco to in order to genetically modify the tobacco chloroplast genome for degradation of RDX and TNT.

### A12.5 Selection of chloroplast-transformed tobacco after bombardment

The leaf discs of tobacco, bombarded with *pLZTNT-aada* or *pLZTNT-ABNR*, were placed on RMOT medium in the dark for two days. Two pieces of bombarded tobacco leaves (5-mm each) were placed on RMOP selection medium with 500 mg/L spectinomycin with the bombarded side in contact with medium for the first round of selection. The Petri dishes were incubated in the culture room under white fluorescent lamps (1,900 lux) with 16 hr light/ 8 hr dark cycle at 26 °C. After 4 to 8 weeks, putative transgenic shoots appeared. Before the second round of selection, 100 mg of leaf material were harvested from the putative transplastomic shoots. DNA was extracted and PCR was used to confirm the integration of the foreign gene in the chloroplast genome. For the second round of selection, 2 mm<sup>2</sup> pieces were cut from the leaves of PCR-positive plants and placed on RMOP selection medium. These leaf sections produced transgenic shoots in 3–4 weeks. Then the regenerated shoots were excised and transferred to MS medium containing spectinomycin. This step was termed the third round of selection, in which rooting occurred in 3 to 4 weeks.

### A12.6 PCR to confirm the integration of RDX- and TNT-degradation genes in the chloroplast genome

DNA samples were isolated from the leaves of plants demonstrating spectinomycin resistance using the Dneasy plant mini kit (Qiagen). PCR reactions were separately primed by 3p+xpla1033R, xpla531F+ xplb1143R, 5P+2M as listed below:

*xpla531F*: GAACAACCCCTATCCCTGGT  
*xpla1033R*: TCAGATAGCCGAAAGCGACT  
*Xplb1143R* - GACTGTCCGGTCGATCACTT  
 3P: AAAACCCGTC CTCAGTTGG ATTGC (anneals to native tobacco chloroplast)  
 3M: CCGCGTTGTTCATCAAGCCTTACG (anneals with the *aada* gene)  
 5P: CTGTAGAACGTACCATTGTTGTGC (anneals with the *aada* gene)  
 2M: TGACTGCCACCTGAGAGCGGACA (anneals to native tobacco chloroplast)

### **A12.7 RT-PCR to confirm the expression of RDX- and TNT-degradation genes in tobacco chloroplast**

Total RNAs were isolated from the leaf discs using the RNeasy plant mini kit (Qiagen). The DNA in RNA samples was digested with DNaseI (Biolab). For reverse transcription, PCR (RT-PCR) analysis of transgene expression was carried out; one µg of total RNA was transcribed into cDNA using MMLV (Invitrogen) and random primers (Invitrogen, 48190-011). One µl of cDNA was used in each PCR amplification. The primers for RT-PCR are listed below:

*xplA531F*: GAACAACCCCTATCCCTGGT  
*xplA1033R*: TCAGATAGCCGAAAGCGACT  
*XplB-511F* - GAATTCA CGGTCCGATCT  
*XplB1143R* - GACTGTCCGGTCGATCACTT  
*PSBAF1*: CTTCATTGCTGCTCCTCCAG  
*PSBAR1*: AGTGCTGATA CCTAAAGCGGT  
*aada078F*: GACGTTGCTGGCCGTACATT  
*aada465R*: ATCAAAGAGTTCCCTCCGCCG  
*NRF3*: ATGAATTCATGGATATCATTCTGTCGC  
*NRR3*: ATGAATTCTCAGCACTCGGTACAATC

### **A12.8 Western blots to confirm accumulation of XplA protein in the chloroplast of tobacco**

Green and healthy leaves were collected from transformed and untransformed plants growing in the greenhouse. The leaf material was ground to a fine powder in liquid nitrogen. Freshly prepared PEB (200 ml of 100 mM sodium chloride, 10 mM EDTA pH 8.0 , 200 mM Tris-HCl, pH 8, 0.05% vol/vol Tween-20, 0.1% wt/vol SDS, 14 mM β-mercaptoethanol , 200 mM sucrose and 2 mM PMSF) was added to each 100-mg plant sample on ice. The homogenized samples were centrifuged at 15,000 g for 10 min at 4°C. The supernatant was retained and the total soluble protein quantified. The protein solution samples were loaded on 10% SDS-PAGE and separated by electrophoresis. The proteins were transferred to PVDF membrane. Rabbit polyclonal anti-*xplA* was used as the primary antibody and the goat anti rabbit IgG, conjugated to horseradish peroxidase (HRP) was used as the secondary antibody.

### **A12.9 Uptake of RDX by chloroplast-transformed tobacco with *xplA-xplB-nfsI***

The *pLZTNT-ABNR* transformed tobacco strains *abnr-22*, *abnr-26* and *pLD-xplA* transformed strain *xplA-5* were cultured in liquid ½MS amended with 180 µm RDX. The concentration of RDX in medium was determined after 2, 5, and 7 days culture. Sampling was repeated three times. Wild-type tobacco was used as a negative control and nuclear genome transformed Arabidopsis with *xplA-xplB-nfsI* was used as the positive control. After seven days' culture, RDX was extracted from the plant samples and analyzed by the following protocol. Samples of leaf (500 mg) was collected from each plant sample, placed in 2 ml tubes with stainless steel balls, and ruptured by shaking in Fast Prep machine. Methanol (1 ml) was added and the tube vortexed. The tubes were shaken at 100 rpm overnight and centrifuged at 15000 for 10 min. The supernatant (900 µL) was pipetted to new tube and centrifuged again for 10 min prior to transfer of 800 µL of supernatant to a 1 ml amber vial for HPLC analysis.

### **A12.10 Construction of the *pLD-ctv2-xplA* vector and transformation of the chloroplast of tobacco**

The *pLD-ctv2* vector was a gift from Professor Daniell of University of Central Florida. This vector has previously been shown to work well for expression of foreign genes in the chloroplast of tobacco. The *xplA* gene was inserted into the *pLD-ctv2* vector as a *NotI*

fragment to produce the *pLD-xplA* vector. This vector was used to bombard tobacco and as a control for our vector system.

### A12.11 Results

The promoters, 5'UTR, and terminators were amplified from the chloroplast genome of tobacco and combined to produce five chloroplast expression vectors referred to as *pLZ1-pLZ5*. One advantage of this vector system is that the promoters, 5UTRs, and terminators in different *pLZ* can be interchanged to produce a variety of modified *pLZ* for enhance foreign gene expression. Another advantage is that expression cassettes containing foreign genes released from different *pLZ* vectors can be inserted into a single target vector. Thus the translation efficiency of every gene is enhanced since every gene is flanked by a different 5'utr and a different terminator.

### A12.12 Expression of the *GUS*, *GFP* and *aada* genes in *E.coli* to confirm the function of expression cassettes of *pLZ1-pLZ5*

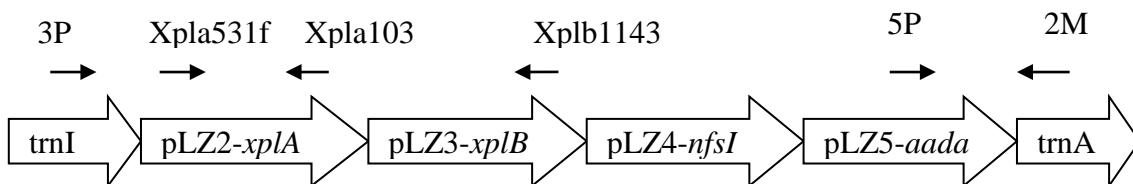
The CDS sequences of *GUS*, *GFP*, and *aada* were constructed into *pLZ1-pLZ5* separately and transformed into *E.coli* strain DH5 $\alpha$ . The DH5 $\alpha$  (*pLZn-GUS*) and DH5 $\alpha$  (*pLZn-GFP*) were cultured on solid LB medium with ampicillin at 37 °C overnight. The DH5 $\alpha$  (*pLZn-GUS*) displayed blue colour on the LB medium supplied with 2 mm x-gluc compared to the white colour of DH5 $\alpha$  harbouring the *pLZ* vector. Similarly, the DH5 $\alpha$  (*pLZn-GFP*) displayed green fluorescence when exposed to UV light. The DH5 $\alpha$  strains harbouring *pLZ-aada* survived and grew in liquid LB medium amended with ampicillin and spectinomycin.

### A12.13 Transient expression of *GFP* gene in chloroplast of tobacco bombarded with *pLZ-GFP*

The transient expression of *GFP* gene in tobacco chloroplasts was carried out by bombarding leaf discs with the *pLZ-GFP* vector. The bombarded leaf discs were cultured on RMOT medium for two days and observed under a fluorescence microscope for the *GFP* signal.

### A12.14 The construction of the target vector *pLZTNT* and the transformation vector *pLZTNT-ABNR*

The full length of 16s rDNA and *trnI* genes with the flanking DNA region was amplified as the upstream homologous region in the target vector. The DNA sequence of *trnA* including the flanking sequence at both ends was amplified as the downstream homologous region. The upstream homologous region, the downstream homologous region, and a linker including five 8 base-pair restriction enzyme recognizing sequences were inserted into *pGEM5zf* to produce target vector *pLZTNT*. The degradation genes *xplA*, *xplB*, *nfsI*, and the selection marker gene *aada* were inserted into *pLZ* vectors separately to produce *pLZ2-xplA*, *pLZ3-xplB*, *pLZ4-nfsI*, and *pLZ5-aada*. The expression cassettes of these genes were then excised using the corresponding 8 base-pair restriction enzyme and inserted into the target vector of *pLZTNT* to produce the transformation vector *pLZTNT-ABNR* (A12.1)



**Figure A12.1. Tobacco chloroplast transformation vector, *pLZTNT-ABNR*.**

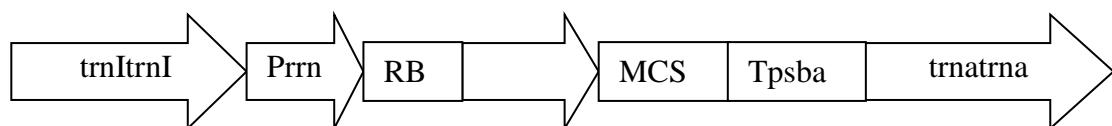
The *xplA*, *xplB*, *nfsI* and *aada* genes were inserted into the *pLZ2-5* vectors. The expression cassettes for these genes were then excised and ligated into the tobacco targeting vectors to produce the transformation vectors.

#### A12.15 Transformation of tobacco chloroplasts with RDX and TNT degradation genes

The construct *pLZtnt-aada* was used to bombard the leaf discs of tobacco and transformed plantlets were regenerated from leaf discs on selection medium. PCR and RT-PCR performed on the regenerated plantlets demonstrated that the *aada* gene was successfully inserted in the chloroplast genome and that it expressed well, conferring resistance to spectinomycin. This result demonstrated that our vector systems were suitable for the transformation of tobacco chloroplast (data not shown). The leaf discs of tobacco were bombarded with *pLZTNT-ABNR* and screened on RMOT medium with spectinomycin at 500 mg/L. After selection for two months, two plantlets were regenerated from leaf discs. PCR for the *xplA* gene demonstrated that one of the plantlets was transformed. The PCR positive plant was used as input for the second and third round selections. After three rounds selection, PCR, RT-PCR, and western blots were carried out to confirm the integration, transcription, and translation of genes in tobacco chloroplasts.

#### A12.16 The construction of the chloroplast transformation vector *pLD-xplA* and the transformation of tobacco by the particle biolistic method

The *xplA* gene was inserted into the *NotI-XbaI* site of *pLD-ctv2* to produce the *pLD-xplA* vector (Figure A12.2). Tobacco leaf discs were bombarded with the *pLD-xplA* vector. After three rounds of screening, ten spectinomycin resistant plants were selected for molecular biology analysis.



**Figure A12.2. The structure of *pLD-xplA***

#### B. List of scientific/technical publications produced during the course of the project

**Rylott, E.L., Jackson, R.G., Edwards, J., Womack, G., Seth-Smith, H.M.B., Rathbone, D., Strand, S.E. & Bruce, N.C.** (2006) An explosive-degrading cytochrome P450 activity and its targeted application for the phytoremediation of RDX. *Nature Biotech.* 24:216-219

**Jackson, R.G., Rylott, E.L., Fournier, D., Hawari, J. & Bruce, N.C.** (2007) Exploring the biochemical properties and remediation applications of the unusual explosive-degrading P450 system XplA/B. *PNAS* 104:16822-16827

**Travis, E.R., Hannink, N.K., Van der Gast, C.J., Thompson, I.P., Rosser, S.J. & Bruce, N.C.** (2007) Impact of transgenic tobacco on trinitrotoluene (TNT) contaminated soil community. *Env. Science & Technol.* 41:5854-5861

**Gandia-Herrero, F., Lorenz, A., Larson, T., Graham, I.A., Bowles, D.J., Rylott, E.L. & Bruce, N.C.** (2008) Detoxification of the explosive 2,4,6-trinitrotoluene in *Arabidopsis*: discovery of bifunctional O- and C-glucosyltransferases. *Plant J.* 56:963-974

**Beynon, E.R., Symons, Z.C., Jackson, R.G., Lorenz, A., Rylott, E.L. & Bruce, N.C.** (2009) The role of oxophytodienoate reductases in the detoxification of the explosive 2,4,6-trinitrotoluene by Arabidopsis. *Plant Physiol.* 151:253-261

**Rylott, E.L. & Bruce, N.C.** (2009) Plants disarm soil: engineering plants for the phytoremediation of explosives. *Trends in Biotechnology* 27:73-81

**Sabbadin, F., Jackson, R. Haider, K., Tampi, G., Turkenburg, J.P., Hart, S., Bruce, N.C. & Grogan, G.** (2009) The 1.5-Å structure of XplA-heme, an unusual cytochrome P450 heme domain that catalyzes reductive biotransformation of Royal Demolition Explosive. *J. Biol. Chem.* 284:28467-28475

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**Rylott, E.L., Jackson, R.G., Sabbadin, F., Seth-Smith, H.M.B., Edwards, J., Chong, C.S., Strand, S.E., Grogan, G. & Bruce, N.C.** (2011) The explosive-degrading cytochrome P450 XplA: Biochemistry, structural features and prospects for bioremediation. *Biochim. Biophys. Acta* 1814:230-236

**Rylott, E.L., Budarina, M.V., Barker, A., Lorenz, A., Strand, S. & Bruce, N.C.** (2011) Engineering plants for the phytoremediation of RDX in the presence of the co-contaminating explosive TNT. *New Phytologist* 192:405-413

**Lorenz, A., Rylott, E.L., Strand, S.E. & Bruce, N.C.** (2013) Towards engineering degradation of the explosive pollutant hexahydro-1,3,5-trinitro-1,3,5-triazine in the rhizosphere. *FEMS Microbiol. Lett.* 340:49-54

**Chong, C.S., Sabir, D.K., Lorenz, A., Bontemps, C., Andeer, P., Stahl, D.A., Strand, S.E., Rylott, E.L. & Bruce, N.C.** (2014) Analysis of the xplA/xplB containing gene cluster involved in hexahydro-1,3,5-trinitro-1,3,5-triazine degradation in *Rhodococcus rhodochrous* Strain 11Y. *Appl. Environ. Microbiol.* 80:6601-10

**Gunning, V., Tzafestas, K., Sparrow, H., Johnston, E., Brentnall, A.S., Potts, J.R., Rylott, E.L. & Bruce, N.C.** (2014) Arabidopsis glutathione transferases U24 and U25 exhibit a range of detoxification activities with the environmental pollutant and explosive, 2,4,6-Trinitrotoluene. *Plant Physiol.* 165:854-865

**Johnston, E.J., Rylott, E.L., Beynon, E., Lorenz, A., Chechik, V. & Bruce, N.C.** (2015) Monodehydroascorbate reductase mediates TNT toxicity in plants. *Science* 349:1072-1075

**Rylott, E.L., Gunning, V., Tzafestas, K., Sparrow, H., Johnston, E.J., Brentnall, A.S., Potts, J.R. & Bruce, N.C.** (2015) Phytodetoxification of the environmental pollutant and explosive 2,4,6-trinitrotoluene. *Plant Signal Behav.* Epub 10:1, e977714, doi: 10.4161/15592324.2014.977714.

**Zhang, L., Routsong, R.<sup>1</sup> Nguyen, Q. Rylott, E.L. Bruce, N.C & Strand, S.E** (2016) Expression in grasses of multiple transgenes for degradation of munitions compounds on live fire training ranges. Submitted for publication.